IMMUNOREGULATION OF EXPERIMENTAL AUTOIMMUNE THYROID DISEASE

PANAYOTIS VERGINIS

Immunoregulation of Experimental Autoimmune Thyroid Disease

by

© Panayotis Verginis

A thesis submitted to the

School of Graduate Studies in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

Faculty of Medicine Supervisor: Dr. George Carayanniotis

Memorial University of Newfoundland

December 2005

St. John's Newfoundland

Your file Votre référence
ISBN: 978-0-494-19628-1
Our file Notre référence
ISBN: 978-0-494-19628-1

ABSTRACT

Experimental autoimmune thyroiditis (EAT) can be induced in mice after challenge with thyroglobulin (Tg) in complete Freund's adjuvant (CFA). EAT resembles Hashimoto's thyroiditis (HT) in humans and is characterized by mononuclear cell infiltration of the thyroid gland leading to hypothyroidism.

In the first part of this study, we attempted to delineate pathogenic Tg peptides that encompass dominant epitopes in order to study immunoregulation mechanisms in EAT. Since susceptibility to EAT is under the control of H-2A^k genes, we utilized an algorithm-based approach that predicts peptides with A^k-binding potential. Five Tg peptides were found to induce EAT in CBA/J (H-2^k) mice. In addition, all five peptides were immunogenic at the T cell level and induced production of IL-2 and IFN-γ by peptide-primed LNCs. Although none of these Tg peptides encompass dominant epitopes, they will allow us to study mechanisms involved in the immunoregulation of EAT. Furthermore, one of the five new Tg peptides (a.a. 2596-2608), was pathogenic in H-2^k strains of diverse non-H-2 background but not in mice of q, s, d, and b haplotypes. The above findings provide important information regarding the influence of H-2 and non-H-2 genes in the thyroiditogenic potential of the Tg peptides.

In the second part of this study, we showed that TNF-α-treated, semi-mature DCs pulsed with Tg, but not with OVA antigen suppressed Tg-induced EAT in CBA mice, through the induction of Tg-specific CD4+CD25+ T cells. These $CD4^+CD25^+$ Treg cells produce high levels of IL-10 and inhibit the proliferation of Tg-specific effector cells in vitro. Suppression was shown to be cytokine-independent but cell-cell contact dependent. Additionally, adoptive transfer of the $CD4^+CD25^+$ Treg cells into CBA hosts suppressed Tg-induced EAT confirming the suppressogenic potential of this cell subset. The induction of antigen-specific Treg cells will contribute to understand the mechanisms involved in the immunoregulation of autoimmune diseases.

Finally, we describe the generation of transgenic mice specific for the p2496 pathogenic Tg epitope. The α and β chain of a p2496-specific I-A^s-restricted T cell hybridoma were identified and inserted into TCR expression vectors. Linearized fragments containing the α and β chain genes and the constant chain regions were injected into fertilized oocytes of (SJLxC57BL6) mice and integration of the transgene was monitored by PCR. The p2496–specific TCR transgenic mice will provide an excellent source of p2496-specific naïve T cells that will be used to answer questions on the maintenance of self tolerance. In addition it allow the study of parameters that lead to activation of the autoractive T cells and initiation of EAT.

Acknowledgments

There are many people who have played a pivotal role in shaping my education and my interest in the field of Immunology. First and foremost I would like to thank to my supervisor Dr. George Carayanniotis. George's sense of leadership, scholarship, as well as his meticulous work and demonstration of support have set a goal that I hope to achieve. I am also grateful to Ms. Karen Carayanniotis who provided ample help throughout my graduate training.
I would further like to acknowledge the members of my dissertation committee, Dr. Michael Grant and Dr. Vernon Richardson, along with all other professors in the Immunology Group of Memorial University of Newfoundland for their helpful suggestions and support throughout my graduate school years.
Financial support to undertake my Ph.D. degree, of which this thesis is an integral part, was provided by the Canadian Institutes of Health Research and the School of Graduate Studies at Memorial University of St. John's .

I would also like to thank the many friends that I have made along the way: Stefanos Intzes, Cliff Guy, Sonya MacParland, Trish Mulrooney, Elisavet Liakata, Vaggelis Karras, Mina Karaiskou, Sofia Kanellopoulou, and all the other students at the Immunology Program in the Memorial University of Newfoundland for their conversations, friendship and for tolerating me. In addition, I would like to thank my fellow Master's and PhD students, Yang Dai,

Ph.D., Kerry Barrett, M.Sc., and Haiyan Li, who each helped make my time in the PhD program more fruitful and productive.

I would like to extend my special thanks to my very close friends Stathis, Vasso, Popi, Dimitris, Vasso, Adonis who, although being apart for many years, have always been by my side.

These acknowledgments would not be complete without thanking my family for their support throughout my endless years in school, particularly my brother and sister-in-law Spiros and Dina Verginis for their continuous encouragement. Last but not least, I would like to thank my parents. They have given their unconditional support and they have been strong enough to allow me to leave my native country, to believe and instill confidence in myself.

Table of Contents

List of Tables

List of Figures

Abbreviations

2-ME	2-mercaptoethanol
a.a.	Amino acid
Ab	Antibody
Ag	Antigen
AITD	Autoimmune thyroid disease
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BM	Bone marrow
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CFSE	Carboxy fluorescein diacetate succinimidyl ester
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DCs	Dendritic cells
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Flt-3l	Fms-like tyrosine 3 kinase ligand
Foxp3	Forkhead transcription factor
GD	Graves' disease

GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen
HT	Hashimoto's thyroiditis
I.I.	Infiltration index
i.p.	Intraperitoneal
i.v.	Intravenous
IBD	Inflammatory Bowel Disease
iDCs	Immature dendritic cells
IFA	Incomplete freund's adjuvant
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
kDa	Kilo dalton
LNC	Lymph node cells
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
mDCs	Mature dendritic cells
MHC	Major histocompatibility complex
mRNA	Messenger RNA
mTg	Mouse thyroglobulin

NOD	Non obese diabetic
OVA	Ovalbumin
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PPD	Purified protein derivative
RBC	Red blood cells
RNA	Ribonucleic acid
s.c.	Subcutaneous
S.I.	Stimulation index
SAT	Spontaneous autoimmune thyroiditis
smDCs	Semi-mature dendritic cells
SPF	Specific pathogen free
T3	Triiodothyronine
T4	Thyroxine
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TPO	Thyroid peroxidase
Treg	Regulatory T cells
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone-receptor
Tween 20	Polyoxyethylenesorbitan monolaurate
Tg	Thyroglobulin
mM	Mili Molar
μM	Micro Molar
RT-PCR	Reverse transcriptase polymerase chain reaction

CHAPTER 1

INTRODUCTION

1.1 The thyroid gland

The thyroid gland is located in the neck and secretes hormones necessary for growth and proper metabolism. The thyroid gland consists of two lobes joined together by the isthmus and is organized into spherical cyst-like structures called follicles. The thyroid follicle is the primary unit of the gland in terms of structure and function. It consists of a lumen surrounded by a single monolayer of epithelial cells (follicular cells) and is enclosed by a thin basal membrane. The lumen is filled with a viscous solution, the colloid, that contains a mixture of proteins, mainly thyroglobulin (Tg), which is the precursor of thyroid hormones, as well as other lower molecular weight proteins and albumin (DeGroot 1984).

The basic function of the thyroid is the production of two hormones, thyroxine (T4) and triiodothyronine (T3). Abnormalities of thyroid hormone production and release result in thyroid disorders. Thus, excessive or insufficient release of thyroid hormones leads to the pathological conditions of hyperthyroidism and hypothyroidism, respectively.

1.2 Autoimmune thyroid disease

Diseases in which the pathology is caused by immune responses to self antigens are called autoimmune. They are classified as systemic or organ-specific depending on the distribution of the antigen to which the autoimmune response is directed. Autoimmune thyroid disorders (AITD) belong to the second clasification because the immune response is directed against the antigens within the thyroid. The AITDs include: Hashimoto's thyroiditis (HT), Graves' disease (GD), post-partum thyroiditis, idiopathic myxoedema and atrophic asyptomatic thyroiditis (Volpe 1991). Since the present study focuses on the experimental autoimmune thyroiditis (EAT), the murine model of HT in humans, I will briefly discuss the general features of the disease in man.

1.3 Hashimoto's Thyroiditis

HT is the most common of the various forms of AITDs, affecting people of all ages and both sexes. HT appears more frequently in women of age 40-60 (9-25 times higher) than in men of the same age group (Tunbridge et al. 1977;Vanderpump et al. 1995). In terms of histopathology, the disease is characterized by the presence of inflammatory cells (lymphocytes, plasma cells and macrophages) within the thyroid gland, distorted follicles, and fibrosis

(Volpe 1991). Germinal centers are also present, similar to those observed in secondary lymphoid organs (mainly accumulation of B cells). Thyroid follicles become necrotic and large-size mitochondria are accumulated (Volpe 1991). Among the intrathyroidal lymphocytes, both $CD4^+$ and $CD8^+$ T cell populations have been observed (Canonica et al. 1984); In some studies, immune complexes formed between Tg and its Abs were demonstrated around the basement membrane of the follicular cells (Kalderon and Bogaars 1977). Finally, the intrathyroidal cytokine expression profile in HT revealed expression of a mixed Th1 and Th2 response (Ajjan et al. 1996;Paschke et al. 1994). Antibodies against Tg (anti-Tg) and against TPO (anti-TPO) have been demonstrated in the sera of HT patients by a variety of methods (Weetman and McGregor 1984). However, the antibody levels do not always correlate with the thyroid pathology, since several patients with HT have been found with no circulatory anti-Tg antibodies (Baker, Jr. et al. 1988). In addition anti-Tg antibodies have been detected in 38% of healthy individuals (Kohno et al. 1988).

1.4 Thyroid antigens

1.4.1 *Thyroglobulin (Tg)*

Tg is a large (660-kDa) homodimeric glycoprotein that serves as a prohormone for the synthesis of T4 and T3. It is composed of two identical

subunits, 330-kDa each, joined together through disulfide bonds. Tg is the most abundant protein of the thyroid gland, comprising the bulk of the follicular colloid as well as a part of the thyroid's intracellular material. It is not a sequestered antigen since it is present in the circulation with an average concentration of 5, 10 and 133 ng/ml in humans, mice and rats respectively (Carayanniotis and Rao 1997).

In humans, the Tg gene has been localized in the long arm of the chromosome 8 (Brocas et al. 1985), in mice on chromosome 15 (Taylor and Rowe 1987), whereas in rats on chromosome 7 (Brocas et al. 1985). The complete primary cDNA sequences of human (Malthiery and Lissitzky 1987;van de Graaf et al. 1997;van de Graaf et al. 2001), mouse (Caturegli et al. 1997;Kim et al. 1998), rat (Musti et al. 1986) and bovine (Mercken et al. 1985) Tg have been deduced from their respective mRNAs. Partial sequences of the goat (Van Ommen et al. 1989) and rabbit (Dunn et al. 1987) Tg have also been published. Among these species there is a >70% homology in the primary a.a. sequence (Carayanniotis and Rao 1997) with a high degree of conservative substitutions (Malthiery and Lissitzky 1987;Mercken et al. 1985). Specifically, the first 1177 residues of hTg, comprise of a domain repeated 10 times. Another domain of 14 to 17 residues occurs three times between residues 1417 and 1464 and another repetitive domain occurs five times between residues 1584 and 2167 (Malthiery and

Lissitzky 1987). The C-terminus of the molecule shows no repetitive domains but is homologous to acetylcholinesterase (Malthiery and Lissitzky 1987).

Tg is synthesized on rough endoplasmic reticulum by thyroid follicular cells. It is subsequently transported to the Golgi apparatus where it undergoes several post-translational modifications such as glycosylation, sulfation, and phosphorylation, (Consiglio et al. 1987;Herzog 1986;Malthiery and Lissitzky 1987). Finally the molecule is secreted into the follicular lumen for iodination and storage. Tg has 140 tyrosyl residues of which about 40 can be iodinated and 7 are hormonogenic sites: 4 major designated A-D and 3 minor designated as G, N, and R (de Vijlder and Den Hartog 1998;Lamas et al. 1989). The iodine content of Tg in vivo varies depending on the iodine intake by the thyroid, the species and the physiological condition. In most mammals, it ranges from 0.2 to 1% per molecule, which corresponds to 10-50 atoms of iodine per 660-KDa dimer. The normal physiological range of Tg-iodine content in humans is 0.25-0.5% per molecule (Charreire 1989).

Thyroid hormone synthesis and secretion follows a multistep cascade. Transport of iodide (I^-) into the thyroid is the first step, which is catalyzed by the sodium-iodide symporter (Na^+/I^- S) (Carrasco 1993). In the next step TPO, oxidizes I^- and intramolecular coupling of iodinated tyrosine residues results in the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Dunn et al.

1983). Iodinated Tg is then phagocytosed by thyrocytes and proteolysed to yield thyroxine (T4) and triiodothyronine (T3) which are released into the extracellular fluid (Dunn 1995). Synthesis and release of thyroid hormones is basically under the control of thyroid stimulating hormone (TSH), secreted by the anterior pituitary (Van Heuverswyn et al. 1985).

Tg undergoes other post-translational modifications such as glycosylation, phosphorylation and sulfation (Charreire 1989;Spiro and Bhoyroo 1988). Tg is also highly glycosylated with carbohydrate moieties making up ~ 10% of its mass (Charreire 1989). A variety of biological roles have been attributed to oligosaccharides i.e. they have been shown to affect iodination and hormone synthesis. The role of sulfate residues in the physiological function of Tg is not known.

1.4.2 *Other thyroid antigens*

TPO is involved in two important steps in the biosynthesis of the thyroid hormones: iodination of Tyr residues on Tg, and intramolecular coupling of iodotyrosines, leading to the formation of T3 and T4. TPO is a transmembrane glycoprotein, of 107 kDa in size and 933 a.a. in length, that is expressed on the cell surface (apical membrane) of the thyrocytes. It is encoded by a gene on chromosome 2 and 12 in man and mouse, respectively (Kimura et al. 1987;Kotani

et al. 1993). The TPO expression is regulated by TSH and involves cyclic AMP production.

Several functions of the thyroid cells are regulated by the binding of the TSH to its receptor (TSH-R). The deduced 764 amino acid sequence of the TSH-R showed that is a G-protein-coupled receptor (Nagayama et al. 1989). The first 415 amino acids encode a large extracellular domain while the remaining 349 constitute the 7-transmembrane domain and the intacellular cytoplasmic tail (Misrahi et al. 1990).

The TSH-R is the major autoantigen in GD, where autoantibodies against the receptor compete with TSH for binding and stimulate cAMP production (Vassart and Dumont 1992). That activates the thyrocytes continuously and leads to excess of thyroid hormone production and hyperthyroidism.

1.5 Animal models of EAT

Animal models have been extensively used to address questions on the mechanisms involved in EAT pathogenesis. The mouse model provides a system whereby both the immunologic and pathogenic features of thyroid disease can be studied. Mice can be manipulated by experimental procedures that cannot be employed in humans such as thymectomy, thyroidectomy or adoptive transfer of cells or serum. Finally inbred mouse strains allow the use of large numbers of

virtually identical animals, leading to valid statistical analysis. Both spontaneous and induced experimental models exist for HT, and their features will be described in the following paragraphs.

1.5.1 *Spontaneous animal models of thyroiditis*

Spontaneous autoimmune thyroiditis (SAT) has been reported in Obese strain (OS) chicken, non-obese diabetic (NOD) mice as well as in Bio-breeding (BB) and buffalo (BUF) rats (Charreire 1989). A few of the studies performed on these spontaneous thyroiditis models are described below.

The best studied spontaneous model of thyroiditis that closely resembles the human autoimmune disorder, in clinical, histopathological endocrinological and immunological aspects, is the OS chicken (Wick et al. 1982). The thyroids of OS chickens exhibit mononuclear cell infiltration, and the presence of germinal centres after 3 weeks of age (Wick et al. 1974). More than 90% of OS chickens have antibodies to thyroid antigens including Tg (>65%), TPO and thyroid hormones (26%) (Wick et al. 1971).

The NOD mouse model exhibits lymphocytic infiltration of both Langerhans islets and thyroid within the 1st and 2nd month of life (Many et al. 1996). The incidence and severity of the disease were shown to be age-dependent and to

vary from colony to colony, from about 18% to > 77% (Bernard et al. 1992). Thyroiditis and autoantibodies to TPO develop in 35% of the mice but the Ab are not reactive to Tg. Recently a NOD MHC congenic strain (designated as NOD.H-2h4) was developed that expresses I-A^k on the NOD background (Braley-Mullen et al. 1999). Thyroiditis developed in 60-70% of 7-10 month old mice drinking normal water, and developed in 100% of mice upon receiving NaI in their drinking water (Braley-Mullen et al. 1999;Rasooly et al. 1996).

Two spontaneous models of autoimmune thyroiditis have been described in rats. The first one was identified by Hajdu and Rona, who observed that inbred Buffalo male rats at 36 weeks of age exhibited spontaneous histopathological changes of their thyroids comparable to those of HT (Hajdu and Rona 1969). Thyroid pathology was similar to that observed in OS chickens. Further studies indicated that the thyroid damage of BUF rats was associated with elevated and decreased levels of TSH and T4 respectively (Kieffer et al. 1978;Silverman and Rose 1971). A second spontaneous model of autoimmune thyroiditis in rats is the Bio-breeding/Worcester (BB/W) model. Lymphocytic infiltration of the thyroid is observed at the age of 8-10 months in both sexes and in almost 60% of the animals (Sternthal et al. 1981). However the serum levels of T3, T4 and TSH in BB/W rats were found to be at normal values suggesting that thyroid infiltration

by mononuclear cells is not extensive enough to result in thyroid failure (Sternthal et al. 1981).

1.5.2 *Induced models of autoimmune thyroiditis*

EAT was first described in 1956, by Witebsky and Rose (Rose and Witebsky 1956;Witebsky and Rose 1956). They challenged rabbits intradermally with homologous thyroid extract in CFA and the animals developed a mononuclear cell infiltration of the thyroid gland as well as autoantibody production. EAT was later induced, in a variety of animal species such as guinea pigs (Paget et al. 1976), rats (Lillehoj et al. 1981;Rose 1975), monkeys (Pudifin et al. 1977), and mice (Tomazic and Rose 1976) by injection of homologous or heterologous thyroid extract in adjuvant. Subsequent studies in rats demonstrated that more severe thyroid destruction developed in immunized animals when highly purified Tg, prepared by ultracentrifugation on sucrose density gradient, was used for immunization instead of whole thyroid extract (Roitt et al. 1965).

Another method that has been used for EAT induction was based on repetitive injections of homologous Tg or thyroid extract in large doses and in the absent of adjuvant (Elrehewy et al. 1981). Almost 60% of highly susceptible strains of mice (CBA) developed thyroiditis and significant antibody titers were

also determined with Tg specificity (Elrehewy et al. 1981). The same protocol resulted in no thyroiditis when tested in poor responders. Finally, several groups have successfully induced EAT by immunizing susceptible mice with Tg synthetic peptides in CFA (Carayanniotis and Rao 1997). Tg-peptide induced EAT will be described in the following section.

Other approaches of EAT induction: In the mouse model several studies have documented thyroiditis induction, following transfer into naive syngeneic recipients, of lymph node cells (LNC) primed in vivo and boosted in vitro with Tg (Braley-Mullen et al. 1985;Simon et al. 1986). According to another study, in vivo Tg-sensitized T-cells further activated in vitro with concanavalin A (con A), were also able to transfer EAT (Okayasu 1985). Moreover, several studies have shown that peptide-primed-LNC after restimulation in vitro with the immunizing peptide were able to transfer EAT in syngeneic naïve recipients (Carayanniotis and Rao 1997). Another approach that has been used to induce EAT is by transferring Tg-specific T-cell clones or lines into naïve mice (Maron et al. 1983;Romball and Weigle 1987). Similarly, Tg-specific T lymphocytes generated by coculture of Tg-primed spleen cells with syngeneic thyroid epithelial cells (TECs) were able to mediate EAT when injected i.v. into syngeneic recipients (Charreire and Michel-Bechet 1982).

A more severe form of EAT, called granulomatous thyroiditis, is induced in naive recipients by adoptive transfer of mTg-primed spleen cells activated in vitro in the presence of either an antibody specific for the IL-2 receptor (anti-IL-2R mAb) or anti-IFN-γ mAb. As in lymphocytic EAT, $CD4^+$ cells are required for the transfer of granulomatous EAT (Braley-Mullen et al. 1991;Stull et al. 1992).

Induction of EAT in mice has been also demonstrated by transfer of syngeneic dendritic cells (DCs) either pulsed in vitro with Tg or isolated from animals that have been primed with Tg in CFA (Knight et al. 1988). Similarly, DCs that were purified from high responder mice were pulsed in vitro with porcine Tg (pTg). Transfer of the Tg-pulsed DCs into syngenic mice resulted in thyroiditis induction and development of IgG2a antibodies indicating that Th1 cells are mainly activated by pTg-pulsed DCs (Watanabe et al. 1999).

In our laboratory, EAT was induced by immunizing mice with conjugates of Tg and mouse class II MHC-specific mAb (Balasa and Carayanniotis 1993). While the priming of mice with mAb-Tg conjugates in the absence of adjuvant was successful in breaking self tolerance to Tg, as demonstrated by the host IgG-responses against Tg, this method failed to induce mononuclear infiltration of

the thyroid, thereby suggesting lack of activation of Tg-specific autoreactive T cells with pathogenic potential in EAT (Balasa and Carayanniotis 1993).

Another method of EAT induction was developed via the manipulation of T-cell subsets in Wistar rats. Following thymectomy and sublethal total body irradiation (200 rads x 5 times at 2 weeks intervals) the animals developed thyroiditis and Tg-reactive Abs (Penhale et al. 1973). Furthermore they demonstrated that EAT can be prevented by reconstitution of thymectomized rats with viable lymphoid cells from syngeneic rats (Penhale et al. 1976).

Among the models for induction of thyroiditis, the Tg-induced EAT mouse model is the most extensively studied. It is an excellent model for immunogenetic studies due to extensive characterization of the MHC locus. Mice can be easily handled and maintained in large numbers and at a lower cost than any other animal developing EAT. Moreover, there is an enormous variety of mouse-specific reagents such as mAbs specific for surface markers expressed in several cell subsets, which can be used to characterize the phenotype and the function of those cells during the autoimmune response.

EAT induction by direct challenge of mice with Tg requires the optimization of various parameters such as Ag dose, route of Ag administration,

adjuvant, immunization protocol and the time of thyroiditis assessment (Charreire 1989;Shulman 1971).

1.6 Immunogenetics of EAT in mice

1.6.1 *MHC genes*

The fundamental role of MHC genes in EAT susceptibility was first described by Vladutiu and Rose in 1971 (Vladutiu and Rose 1971). In their study they used 33 inbred mouse strains representing 11 different haplotypes. EAT was induced by immunization with thyroid extract in CFA, and mouse strains were classified based on the existence of mononuclear cell infiltration within the thyroid and the extent of its follicular destruction. Specifically, mice carrying the $H\text{-}2^{k,s}$ haplotypes were designated as excellent responders, the $H\text{-}2^{q}$ strains were good, strains with the $H\text{-}2^{a,m,p}$ were fairly good whereas mice of $H\text{-}2^{b,d}$ and $H\text{-}2^{v}$ were poor and very poor responders respectively. In the same study, congenic strains such as C3H.SW ($H\text{-}2^{b}$) and C3H/HeJ ($H\text{-}2^{k}$), that share the same background genes but have different H-2 alleles were classified as low and high responders, respectively. From those studies it was evident that EAT induction in mice is under the genetic influence of MHC genes.

In an attempt to localize the susceptible locus within the MHC complex, intra-H-2 recombinant mouse strains were challenged with mTg and genes

located at the centromeric side of the H-2 region were shown to influence the high anti-Tg response (Tomazic et al. 1974). However, the lack of suitable intra-H-2 recombinant strains, at that time, did not permit precise localization of the gene(s)that predispose to susceptibility. Further studies based on new intra-H-2 recombinant congenic strains of B10 background with various combinations of k, b, q alleles at the K and/or I-A regions, localized the disease susceptibility to the I-A locus of the H-2 complex (Beisel et al. 1982a). The significance of the I-A locus in EAT susceptibility was confirmed when mice that have been treated with anti-I-A mAbs either before or at the time of antigenic challenge with Tg in CFA were unable to develop EAT (Vladutiu and Steinman 1987a).

Although the I-A is the major locus conferring susceptibility in mouse EAT model, K and D gene regions have been shown to influence the severity of EAT. Using several recombinant strains expressing s, k, d and b alleles at the I-A locus, and the same or different alleles at the D locus, it was shown that the D-end genes have a regulatory effect on the disease process (Kong et al. 1979). Like the D-end, the K- end has also been shown to influence the severity of the disease. Experiments performed using strains of mice with point mutations at the H-2K gene showed that a low responder mouse can become a high responder upon the introduction of the mutation (Maron and Cohen 1979). A second line of evidence implicating the K-end locus in regulating the severity of EAT was

shown in studies of intra-H-2 recombinant mice (Beisel et al. 1982a). In these studies it was shown that expression of K^k reduces, whereas the presence of K^q or K^b increases the incidence of thyroiditis in mice.

In contrast to the strong association of EAT susceptibility with the H-2 locus, studies on HLA association with HT in patients have resulted in controversy (Weetman and McGregor 1994). Family studies have failed to establish a linkeage of HT and HLA (Roman et al. 1992). That was primarily due to the serologic typing of only DR antigens following the identification of linkage disequilibrium between certain DR and DQ genes. Other parameters that contributed in this controversy wre the differences among populations, the multiple epitopes that possibly exist on Tg, and the involvement of other thyroid antigens during HT development (Weetman and McGregor 1984). However, other studies demonstrated an association of goitrous HT with HLA DR5 (Farid et al. 1981) and atrophic HT with HLA DR3 (Moens et al. 1978). Recently, in an attempt to define the HLA class II molecules associated with autoimmune thyroiditis, transgenic mice have been generated to express several HLA molecules in the absence of other class II genes (Kong et al. 1997). EAT was induced by immunization of transgenic mice with human or mouse Tg in adjuvant. Based on these studies it has been shown that DR3 and DQ8 alleles influence susceptibility whereas DR2, DR4 and DQ6 alleles influence resistance

to EAT induction with hTg (Kong et al. 1996;Kong et al. 1997;Wan et al. 2002). Immunization with mTg resulted in EAT only in DR3 mice but not in DQ8. In order to examine the effects of multiple class II genes as seen in humans, a more recent study used class II negative strain of mice that co-express DR3 and DQ8 HLA molecules (Flynn et al. 2002). Upon mTg challenge, EAT severity was significantly reduced in DR3/DQ8 mice compared with the single DR3 mice (Flynn et al. 2002). The mechanisms involved in this regulation remain to be determined. In summary, the use of single and double HLA transgenes may help in addressing questions associated with the HLA contribution to susceptibility or resistance in thyroid autoimmunity.

1.6.2 *Non-MHC genes*

The contribution of the non-MHC genes to development of thyroiditis has been studied using congenic mouse strains carrying the same H-2, but different background genes. The mice were immunized with mTg and 3 hours later they received LPS (Beisel et al. 1982b). In this study it was found that genes outside the MHC locus influence the severity and the incidence of thyroiditis, as well as the levels of the Tg-specific antibodies. For example, when the B10, BALB.B, C3H.SW and A.BY mice (all of those carry the H-2^b haplotype) were compared it was shown that C3H.SW and A.BY mice developed higher mTg-specific antibody levels and higher incidence of thyroiditis than B10 or BALB.B mice. It

was suggested that the non-MHC genes of the C3H strain favored EAT development. Similar results were obtained when the B10.BR, BALB.K and C3H/Anf strains that carry the high responder haplotype (H-2^k) were compared. Another study, using recombinant inbred strains, suggested that genes from the Igh locus have an effect on the levels and the subclass of anti-Tg antibodies (Kuppers et al. 1994). Following immunization of CBA-Tu (Igh^j) and CBA-Igh^b recombinant strains with mTg in CFA, it was shown that the Igh^b haplotype produces very low levels of IgG2a mTg-specific antibody compared with the Igh^j haplotype (Kuppers et al. 1994).

In a more recent study it was demonstrated that Tg gene is a major susceptibility gene in human autoimmune thyroid disease (Tomer et al. 2002). This study was based on the analysis of 102 families by using Tg microsatelites and provided strong evidence for the association of Tg gene locus with the susceptibility to thyroid disease.

1.7 Mapping of pathogenic Tg T-cell epitopes

1.7.1 *Overview of the existing knowledge of Tg T-cell determinants*

Although a large body of evidence supports the crucial role of T cells in both the inductive and the effector stages of Tg-induced EAT, very little is

known about the exact mechanisms involved in the development of the disease. A major obstacle in this direction has been the large molecular size of Tg. Tg, as a large macromolecule, is likely to encompass several pathogenic T-cell determinants that are recognized by distinct T-cell populations that may be involved in EAT development. In addition, Tg circulates at low levels in the bloodstream (Torrigiani et al. 1969) and it has been hypothesized that participates in tolerogenic mechanisms established to protect against autoreactivity (Lewis et al. 1987;Lewis et al. 1992;Rayner et al. 1993). Therefore, in order to study mechanisms leading to EAT development, it was necessary to develop a simplified model in which T-cell subsets of several specificities would be eliminated, and disease induction by pathogenic Tg T-cell subsets could be easily followed. As a result, interest was focused on the mapping of pathogenic T-cell sequences of Tg.

T-cell determinants have been classified as immunodominant, subdominant or cryptic (Sercarz et al. 1993). An immunodominant determinant strongly stimulates the in vitro proliferation of T cells activated in vivo with intact antigen whereas a cryptic determinant fails to do so (Moudgil and Sercarz 1993). Both immunodominant and cryptic determinants can successfully stimulate T cells isolated from mice that have been immunized with the respective determinant in adjuvant. On the other hand, an epitope can be

classified as subdominant when it can induce proliferative cells that respond in vitro to both the determinant itself and to the native antigen. T cells induced by immunization with the native antigen respond inconsistently in vitro to the subdominant determinant.

Several investigators from different laboratories have focused on identifying pathogenic, Tg epitopes within the large Tg molecule. Diverse strategies have been employed for the mapping of pathogenic epitopes. One of them is based on the generation of Tg-specific T-cell hybridomas and subsequent use of these clones as tools for screening for Tg T-cell epitopes with thyroiditogenic potential (Champion et al. 1991;Texier et al. 1992). A second strategy uses computerized algorithms to predict potential T-cell epitopes within the Tg sequence and the subsequent testing of the candidate sequences for their ability to induce disease in animals (Carayanniotis et al. 1994;Chronopoulou and Carayanniotis 1992).

Following the first approach, two T-cell epitopes have been identified; the first epitope is a 9-mer peptide corresponding to a.a. (2551-2559) of hTg and containing T4 at position 2553 (Champion et al. 1991). Its identification was based on the use of two CH9 and ADA2 hybridoma clones that were activated by the Tg fraction carrying T4 and their activation was dependent on the iodination

level of that fraction (Champion et al. 1987). Since there are only four hormonogenic sites on Tg at residues 5, 2553, 2567 and 2746 (Malthiery and Lissitzky 1987) an array of 5 to 12-mer overlapping synthetic peptides covering the four hormonogenic sites was synthesized and each peptide was tested for its capacity to activate the two hybridomas. In this way the minimal T cell epitope was mapped to the C-terminus of Tg including the 2553 residue (Champion et al. 1991). The (2551-2559) peptide did not induce thyroiditis in CBA/J mice after direct challenge with CFA. However, lymph node cells (LNC) isolated from mice that had been primed either with intact Tg or with the (2551-2559) peptide activated effector cells that successfully transfered thyroiditis to naive syngeneic recipients following in vitro stimulation with (2551-2559) peptide (Hutchings et al. 1992). An obvious question arising from this study was whether the presence of iodine atoms was critical for T cell activation. To address this Kong and co-workers tested three hTg 12mer peptides (1-12, 2549-2560, and 2559-2570), carrying T4 at the other three hormonogenic sites 5, 2553 and 2567 (Kong et al. 1995). Peptides carrying thyronine (T0, lacks the four iodine atoms) were synthesized and used as controls. It was found that (2549-2560) peptide containing either T4 or T0 was immunogenic at T-cell level and splenocytes isolated from CBA mice primed either with mTg or with the respective peptide and re-stimulated in vitro with peptide, were able to adoptively transfer disease

to syngeneic naïve mice. These data suggested that the presence of iodine was not necessary for activation of the autoreactive T cells and disease development. On the other hand, both T4(5) and T4(2567) peptides were slightly immunogenic and only the T4(5) was able to generate mild thyroiditis (Kong et al. 1995). Similarly, the peptide 2737-2748 that contains the T4 at position 2746 was not found to be immunogenic suggesting that the presence of iodine molecules within the peptide structure is not sufficient to render the peptide immunogenic (Carayanniotis and Kong 2000).

The second Tg T-cell epitope identified using the hybridoma-based strategy,was a 40-amino acid sequence localized between residues 1672 and 1711 of hTg (F40D) (Texier et al. 1992). Initially it was found that a <10kDa porcine Tg tryptic fragment was able to induce thyroiditis in CBA mice (Salamero et al. 1987). By using an MHC class I-restricted cytotoxic T lymphocyte (CTL) hybridoma (HTC2) that was generated by immunization of mice with pTg it was shown that syngeneic macrophages, pulsed with pTg tryptic fragments, were lysed in a dose-dependent manner. To identify the sequence(s) responsible for the activation of HTC2 cells the <10 kDa fraction of pTg was separated by 2D-gel electrophoresis. Those products of electrophoresis that could successfully activate the HTC2 cells were collected and further purified by high-performance

liquid chromatography (HPLC). HPLC fractions coresponding to the major peak that could activate the HTC2 cells were used for N-terminal sequencing. Following this procedure the F40D sequence was defined that could induce lymphocytic thyroiditis in 4 out of 5 mice, after direct subcutaneous challenge (Texier et al. 1992). It is possible that this 40-mer fragment contains more than one pathogenic sequence but complete epitope mapping has not been carried out.

Our laboratory made use of computerized algorithms (Margalit et al. 1987;Rothbard and Taylor 1988) to scan the rTg (mTg sequence was unknown at that time). A 17mer peptide (2495-2511) was identified to be pathogenic in H-2^k (B10.BR, C3H) and H-2^s (SJL) but not in H-2^d (BALB/c) or H-2^b (B10) strain of mice (Chronopoulou and Carayanniotis 1992), indicating that the MHC susceptibility to EAT induced by this peptide was similar to the pattern of the mTg-induced EAT (Vladutiu and Rose 1971). The Tg epitope(s) in this peptide was not dominant because: a) it failed to stimulate Tg-primed LNCs in vitro; and b) peptide-primed LNCs were not activated in the presence of Tg in culture. Amino acid (a.a.) truncation analysis revealed two pathogenic T-cell epitopes within (2495-2511) (Rao et al. 1994). The first one was a 9-mer rTg (2946-2504) epitope that elicited T-cell proliferative responses as well as severe EAT in both H-2^k and H-2^s mice. The second one was a 8-mer rTg (2499-2507) that induced mild EAT in

both k and s strains of mice but was not able to activate peptide-primed LNCs in vitro (Rao et al. 1994).

Via the same algorithm approach, a second peptide on rTg (2695-2713) was identified and tested for EAT induction in several mouse strains (Carayanniotis et al. 1994). The peptide elicited EAT in SJL ($H\text{-}2^s$) but not C3H or B10.BR ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$) and B10 ($H\text{-}2^b$) mice (Carayanniotis et al. 1994). These findings suggested a new genetic pattern of susceptibility to EAT (different from that of Tg-induced EAT). This peptide was also non-dominant since priming of SJL mice with rTg was unable to elicit T-cell responses to the respective peptide in vitro (Carayanniotis et al. 1994).

Recently, a 20-mer hTg peptide (2340-2359) was identified to contain multiple E^k-binding motifs (Altuvia et al. 1994;Leighton et al. 1991). Initially, the peptide had been identified to be recognized by Tg-reactive autoantibodies in sera from patients with Graves' disease (Thrasyvoulides et al. 2001). The peptide was shown to contain multiple I-E^k binding motifs upon scanning using a computer algorithm (Altuvia et al. 1994) and, therefore, it was tested for pathogenicity in $H\text{-}2^k$ mice. The peptide was immunogenic at both the T- and B-cell level and peptide-specific T cells mediated EAT in AKR/J mice ($H\text{-}2^k$) (Karras et al. 2003). Blocking studies using anti-I-E^k mAbs confirmed I-E^k binding-motifs

in the pathogenic sequence whereas anti-I-A^k mAbs failed to block the proliferation of peptide-primed T cells.

A third approach used for the identification of pathogenic Tg peptides was based on earlier findings by McLachlan and Rapoport (McLachlan and Rapoport 1989) that hTg and hTPO share B-cell epitopes and it was hypothesized they may also share T-cell epitopes. Based on this study Hoshioka and co-workers (Hoshioka et al. 1993) identified two 14-mer peptides, the hTg(2730-2743) and hTPO(118-131), which share 5 identical consecutive a.a. However, direct challenge of CBA mice with the hTg peptide failed to induce EAT and similar result was obtained after adoptive transfer of peptide activated T cells. Extensive lymphocytic infiltration of the thyroid was observed only by adoptive transfer of cells that were primed in vivo with mTg and boosted in vitro with hTg(2730-2743). The reasons for this discrepancy were not discussed in the paper.

Based on the above discussion it is evident that mapping of pathogenic Tg epitopes it is a difficult task. One of the projects in the current study focuses on the identification of pathogenic Tg epitopes through an algorithm approach. I will proceed therefore to discuss how algorithms that predict T cell epitopes or MHC ligands have been formulated and used.

1.7.2 *Algorithm based prediction of T-cell epitopes*

In order for T cell activation to take place the T cell receptor must recognize antigenic peptides presented in the context of the MHC on the APC. It was therefore important to discover specific features of antigenic sequences (T-cell epitopes) that facilitate their binding to certain MHC molecules. It was initially proposed that the hydrophobic portions of the antigenic determinant interacts with the APC whereas the hydrophilic portion confers specificity on the interaction with the TCR (DeLisi and Berzofsky 1985). In this way the antigenic site postulated to form an amphipathic helical structure characterized by both hydrophilic and hydrophobic portions. Considering the predisposition of antigenic sites to form amphipathic structures, Margalit and colleagues developed an algorithm that could predict potential T cell epitopes within a protein (Margalit et al. 1987). In another study, Rothbard and Taylor analyzed the primary sequence of 57 known helper and cytotoxic T cell determinants in an attempt to delineate common motifs (Rothbard and Taylor 1988). Two motifs described; the first consists of four residues whereas the second consists of five residues. Within the 57 T-cell epitopes tested the tetramer motif was identified in 46 (81%) and the pentamer in only 18 (32%). Both approaches described above can be used either to search for antigenic sites in any protein sequence regardless of their MHC restriction or to identify determinants that bind to limited MHC

alleles. It was necessary therefore, to identify specific motifs that allow peptides to bind to certain MHC molecules. For MHC class II ligands the prediction of peptide binding motifs was found to be more difficult due to the variable length of the ligands that can be accommodated in the MHC groove as well as the more degenerate anchor positions.

To date several algorithms have been developed with the ability to predict epitopes that can bind to various MHC class I and II molecules, based either on the analysis of natural MHC ligands or on the binding properties of synthetic peptides (Chicz et al. 1992;Falk et al. 1994;Hunt et al. 1992a;Hunt et al. 1992b;Leighton et al. 1991;Rudensky et al. 1992). In that regard, structural motifs have been described for non-self sequences that can activate T cells in the context of MHC class II alleles and for self-peptides that have been eluted from purified MHC class II molecules. On the basis of those studies MHC class II-binding motifs have been reported for I-A^k, I-E^k, I-A^d, I-E^d, I-A^s, I-E^b, I-A^b, DR1, DR2, DR3, DR4, DR7, DR11, DQ2, and DQ8 (Rammensee et al. 1999;Rammensee et al. 1995).

Since susceptibility to Tg-induced EAT is controlled by genes in the H-2A region and mice bearing the k haplotype are characterized as "excellent responders", we were interested in performing a more systematic search for peptides that can bind to I-A^k molecules. In the next paragraphs I will focus on algorithms that describe I-A^k binding motifs.

The first direct evidence for an I-A^k binding motif was described on studies with the hen egg lysozyme (HEL) (Allen et al. 1987). The authors utilized two I-A^k–restricted T cell hybridomas specific for the (46-61) peptide of the HEL (Allen et al. 1984). Epitope mapping study revealed that the minimal epitope was encoded by the residues 52-61 (Allen et al. 1985;Babbitt et al. 1985). Amino acid substitution analysis within the minimal epitope determined that I-A^k –binding required three a.a. residues (at positions 1, 7 and 10). Subsequent alignment between several naturally processed peptides extracted from I-A^k molecules on APC and the HEL peptide revealed a pattern for the three anchor positions described above (Allen et al. 1987;Nelson et al. 1996).

In another study, the crystal structure of the I-A^k molecule in combination with the dominant epitope HEL (50-62) was published (Fremont et al. 1998). Structural analysis of the peptide-I-A^k complex, revealed that the peptide is bound deep into the A^k groove and it extends upward at the C-terminal end. Five peptide binding pockets at positions 1,4,6,7 and 9 as well as the residues that each pocket can accommodate were delineated (Fremont et al. 1998). Combination of this information resulted in a more restrictive motif for peptide binding to I-A^k molecule. Moreover, the description of the I-A^k anchor residues and the TCR contacts described in this study are in agreement with those delineated in previous studies (Allen et al. 1987).

Following another approach, Altuvia and co-workers developed a computerized algorithm that describes peptide properties for binding to A^k and E^k molecules (Altuvia et al. 1994). The algorithm was constructed by taking into account shared features of peptides that were known to bind to A^k or E^k molecules and elicit T cell responses. Peptides that could not bind to those molecules were also analyzed as controls. The motifs were based on the physical-chemical and structural properties, such as size, hydrophobicity, charge, hydrogen bonding capability etc., of the a.a. that could be accommodated in a specific position within the peptide sequence. Similar to previous studies (Itoh et al. 1996;Nelson et al. 1996), certain positions critical for peptide binding to the MHC were also described (Altuvia et al. 1994).

1.8 TCR transgenic mice

In the past, studies of organ-specific autoimmunity have been limited to those animals that spontaneously developed disease or to those diseases that occur in animals following the injection of autoantigens in adjuvant. With the generation of TCR transgenic mouse technology, new opportunities for studying autoimmune mechanisms were provided. Specifically, T cell receptor (TCR)-transgenic mice have been used to address questions in basic mechanisms of self-

tolerance and to understand the roles of immunological, environmental and genetic factors in the generation, activation and expansion of self-reactive T cells.

The major advantage of TCR-transgenic mice is that one can isolate large numbers of homogeneous naïve T cells with a unique specificity (von Boehmer 1990). Several TCR transgenic lines have been generated that recognize self-antigens involved in T-cell mediated autoimmune diseases such as EAE (Goverman et al. 1993), type I diabetes (Katz et al. 1993) and autoimmune gastritis (Alderuccio et al. 2000). In the next paragraphs, I will discuss how such TCR transgenic mice have been utilized to address mechanisms that lead to the breakdown of tolerance in the periphery and trigger the initiation of autoimmune disease.

1.8.1 *TCR transgenic mice as models of autoimmune disease.*

EAE is one of the best-characterized models of an organ-specific T-cell mediated autoimmune disease (Zamvil and Steinman 1990). The disease is induced in susceptible animals upon immunization with antigens normally presented in the central nervous system (CNS), such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin olygodendrocyte glycoprotein (MOG), in adjuvant. About one to two weeks after immunization, the animals

are characterized by perivascular infiltration followed by demyelination in the brain and the spinal cord (Goverman 1999). The chronic form of the disease bears some resemblance to multiple sclerosis in humans (Goverman 1999).

Two laboratories have produced TCR transgenic mice in order to study the development, tolerance induction and pathogenic potential of CD4+ T cells in EAE (Goverman et al. 1993;Lafaille et al. 1994). These mice (H-2^u haplotype) express the α and β chains of an MHC class-II restricted MBP (1-11)-specific TCR transgene on >90% of their T cells. The fact that large numbers of mature CD4+ T cells expressing the transgenic TCR developed in mice of the H-2^u haplotype indicates that MBP (1-11)-specific T cells are not negatively selected despite the fact that MBP is a self-antigen. The transgenic T cells were able to proliferate and secrete IL-2 in response to exogenously added MBP peptide indicating that the T cells are not anergic (Goverman et al. 1993).

One of the most interesting observations made in this model was that spontaneous EAE was seen only in mice that were housed under conventional conditions and was not observed when the TCR transgenic mice were housed under specific-pathogen-free conditions (Brabb et al. 1997;Goverman et al. 1993). This observation suggests that exposure to microbes or other environmental factors may trigger the initiation of the disease. It is also indicates the presence of

a regulatory mechanism that prevents the activation of the transgenic cells in mice kept in pathogen-free conditions.

To exclude the participation of non-transgenic T cells or other components of the immune system in this regulation, Lafaille et al. crossed the MBP transgenic mice (T/R+) with RAG-1-deficient mice (T/R-) (Lafaille et al. 1994). In this model all mice developed spontaneous EAE. The authors concluded that EAE can be mediated by MBP-specific $CD4^+$ T cells in the absence of other T cells and also that in T/R+ mice an undefined lymphocyte population can protect against spontaneous EAE (Lafaille et al. 1994). To define this cell population, two groups systematically examined several lymphocyte populations by crossing T/R+ mice with mice that lack B cells (IgM-μ KO), $CD8^+$ T cells, NKT cells and γδ T cells (β2m KO) (Olivares-Villagomez et al. 1998;Van de Keere F. and Tonegawa 1998). In all the transgenic-KO mice described above, the incidence of spontaneous EAE was low as it was in T/R+ mice. In contrast, when T/R+ mice crossed with CD4 KO mice or αβ KO mice, significant EAE was developed. This indicates that $CD4^+$ T cells must be responsible for the protection observed in TCR transgenic mice. The ability of $CD4^+$ T cells to protect against EAE was confirmed by adoptive transfer of such cells, purified from normal mice, into transgenic RAG-1 deficient mice (Olivares-Villagomez et al. 1998;Van de and Tonegawa 1998).

In the field of diabetes, a TCR transgenic line was created using TCR genes derived from a $CD4^+$ clone that was islet-antigen-specific, H-$2g^7$ restricted and diabetogenic (Katz et al. 1993). The transgenic mice (termed BDC2.5) were utilized to study the activation and effector function of autoreactive T cells that lead to destruction of pancreatic β cells. Transgenic T cells in these mice were not clonally deleted in the thymus or in the periphery (Katz et al. 1993). In addition, T cells from the TCR transgenic mice were not anergic, as they could respond quite well to islet cells in vitro and infiltrated the pancreatic islets in vivo. For example, the authors observed that T cells ignore the islet for the first 3 weeks after birth. At that time, T cells up-regulate the expression of co-stimulatory molecules and other mediators involved in lymphocyte homing resulting in a massive infiltration of the pancreas and induction of insulitis (Andre et al. 1996).

Mc Devitt and coworkers generated another TCR transgenic line from an I-Ag7-restricted GAD65 286-300-specific T-cell hybridoma (Tarbell et al. 2002). By using GADp286 /I-Ag7 tetramer stainings they showed that p286-specific CD4+ T cells responded to the peptide in vitro indicating that the cells were not anergic (Tarbell et al. 2002). Although the p286 mouse cell line was generated in the NOD strain, no diabetes was observed and insulitis was only observed occasionally. This was a strong indication for a regulatory role of the GAD65-specific T cells. In a follow up study, adoptive transfer of GAD65-specific

transgenic T cells along with diabetogenic T cells delayed the diabetes development in NOD.scid mice supporting the above hypothesis for the tolerogenic role of the p286-specific T cells (Kim et al. 2004).

The above studies highlight the importance of TCR-transgenic mouse in studying mechanisms that are ivolved in the initiation and progression of autoimmune diseases as well as in identifying immunoregulatory protocols that can lead to disease suppression. In chapter 6, I describe the generation of a Tg peptide-specific TCR transgenic mouse that will allow us to study parameters that can influence the activation of Tg peptide-specifc T cells.

1.9 Dendritic cells

Dendritic cells (DCs) are specialized APCs considered the main subset of cells involved in T cell priming. DCs are produced from hematopoietic stem cells within the bone marrow and migrate to nonlymphoid tissues where they become resident cells (Bell et al. 1999). At this stage, DCs are unable to activate T cells since they present an immature phenotype by expressing low levels of MHC class II molecules (MHC^{low}) and co-stimulatory molecules ($CD80/CD86^{low}$) and neither CD40 nor CD54, two additional important molecules required during T cell activation (Banchereau et al. 2000). However, they are well equipped to capture antigens by several pathways; (i) macropinocytosis (Sallusto et al. 1995),

(ii) receptor-mediated endocytosis through C-type lectin receptors (DEC205) or Fcγ receptors type I and II, (Jiang et al. 1995) and (iii) phagocytosis of particles, apoptotic bodies, viruses, bacteria etc (Banchereau et al. 2000).

Upon recognition and internalization of the antigen, the immature DCs undergo phenotypic and functional changes. The DCs are now able to migrate from the peripheral tissue to the draining lymphoid organs where they lose their ability for antigen capture. During maturation, DCs assemble large numbers of MHC-peptide complexes on their surface and upregulate the expression of CD80/CD86 as well as CD40 co-stimulatory molecules that are necessary for T cell activation (Banchereau et al. 2000;Turley et al. 2000). Moreover, upon stimulation, DCs produce cytokines that depend on the nature of the stimulus. For example, IL-12 a critical Th1-polarizing cytokine, is elicited by most pathogens but is not induced by stimuli such as TNF-α, IL-1 or cholera toxin (Kalinski et al. 1999).

1.9.1 *Dendritic cells and tolerance induction*

A large body of literature provides evidence for the DCs as regulators of autoimmune responses (Jonuleit et al. 2001;Steinman et al. 2003). The major question regarding the DCs and their influence in tolerance is how the same APCs are able to initiate an autoimmune response and also suppress

autoimmune diseases? Moreover, what are the factors influencing the ability of DCs to activate or suppress an autoimmune response?

The mechanisms that are involved in the induction of peripheral tolerance by DCs are still poorly defined. Evidence has been provided to support three mechanisms that may be involved in the tolerogenic properties of the DCs; (i) induction of anergy by immature DCs, (ii) deletion of effector T cells and (iii) induction or expansion of regulatory T cells.

It has been shown that T cells encountering antigen presented by APCs, which express low levels of co-stimulatory molecules, become anergic. In the absence of inflammatory signals, DCs are immature and express very low levels of MHC and low or no co-stimulatory molecules such as CD80/86 and CD40 (Inaba et al. 1994;Mommaas et al. 1995) and therefore are not able to prime naïve T cells. One factor that has been shown to inhibit DC maturation is IL-10 (Lutz and Schuler 2002;Steinbrink et al. 1997). Several groups have demonstrated that treatment of DCs with IL-10 suppresses their abilty to secrete pro-inflammatory cytokines such as IL-1, IL-6 and IL-12 (Buelens et al. 1997;De Smedt et al. 1997;Koch et al. 1996), and also results in the downregulation of MHC class II and costimulatory molecules expression on their surface (Buelens et al. 1995;Chang et al. 1995;Steinbrink et al. 1997). Experimental evidence for the induction of anergic T cells by IL-10-treated DCs was provided by Enk and co-

workers (Enk et al. 1993). DCs were isolated from the skin and treated with IL-10. Cytochrome C-pulsed IL-10-treated DCs were used to activate cytochrome C-specific Th1 T cell clone. T cells were rescued after 1 day of coculture with IL-10-pretreated DCs and restimulated with untreated DCs in the presence of Ag. T cells incubated with IL-10-pretreated DCs were found to be anergic, whereas T cells incubated with untreated DCs proliferated normally after further stimulation (Enk et al. 1993). In another study, human IL-10-treated DCs, from peripheral blood, induced alloantigen-specific anergy in alloreactive $CD4^+$ T cells in vitro, and anergic T cells were characterized by reduced proliferation that could be restored in the presence of IL-2 (Steinbrink et al. 1997).

As mentioned above, DCs may influence tolerance induction via deletion of antigen-specific T cells. Steinman's group demonstrated that presentation of antigen by immature DCs leads to deletion of antigen-specific $CD4^+$ T cells (Hawiger et al. 2001). The authors utilized a mAb specific for the DEC-205 (Swiggard et al. 1995), an endocytic receptor that is highly expressed on DCs, in order to target HEL peptides to DC. When the peptide was delivered in the absence of stimulatory signals, to exclude maturation of DCs, HEL peptide-specific T cells were deleted from the challenged mice leading to tolerance induction. However, injection of anti-CD40 mAb (that causes DCs maturation),

along with DC-targeted antigen led to development of IFN-γ-secreting T effector cells and immunity (Hawiger et al. 2001).

In a parallel vein, OVA conjugated to the DEC-205 mAb was injected subcutaneously into C57BL/6 mice and 48 h later DCs isolated from the draining lymph nodes were examined for their ability to stimulate OVA-specific CD8+ T cells from a TCR transgenic mouse model (OT-I) (Bonifaz et al. 2002). It was shown that T cells were able to divide for 4-7 cycles and were then deleted. When OT-I T cells were adoptively transferred in vivo along with the OVA-DEC-205 it was demonstrated that T cells were deleted after few cycles of proliferation. The mice were subsequently immunized with OVA in CFA but they were completely unresponsive indicating the induction of tolerance (Bonifaz et al. 2002). The above findings suggest that DCs in a steady state induce deletional tolerance, but following their maturation they are able to induce immunity.

The third mechanism accounts for participation of DC in tolerance induction is via generation of regulatory T cells (Tregs). In the next paragraphs I will focus on the regulatory T cells and how DCs have been shown to participate in their induction *in vitro* and *in vivo*.

1.10 Regulatory T cells (Tregs)

There is now compelling evidence for the existence of T cell subpopulations that are involved in the suppression of immune responses and

play an important role in the immune regulation (Shevach 2000). These cells have been termed Treg cells and are characterized as naturally occurring or inducible. It was initially shown that transfer of $CD4^+$ T cells depleted of $CD25^+$ cells in naïve mice, induced multi-organ autoimmunity, such as thyroiditis, gastritis and insulitis (Sakaguchi et al. 1995). Reconstitution of $CD25^+$ cells a short period after the $CD25^-$ cell transfer prevented the development of the autoimmune diseases (Sakaguchi et al. 1995). This finding suggested the existence of a natural-occuring population of T cells that might play a role in the maintenance of self-tolerance. $CD4^+CD25^+$ T cells have since been shown to inhibit autoimmune diabetes in mice (Salomon et al. 2000), and cure inflammatory bowel disease (Mottet et al. 2003;Read et al. 2000). Several studies also support an important role of the $CD4^+CD25^+$ T cells in transplantation tolerance (Hara et al. 2001) and the control of graft-versus-host disease (GVHD) following BM transplantation (Trenado et al. 2003).

Several groups have provided evidence that naturally-occuring Treg cells are generated in the thymus with the ability to recognize self-antigens (Asano et al. 1996;Itoh et al. 1999;Suri-Payer et al. 1998). The signals that are involved in the generation of the $CD4^+CD25^+$ T cells in the thymus are not completely defined. However, interactions between TCRs with self-peptide/MHC complexes expressed on the thymic stromal cells have been shown to play a critical role in

the positive selection of $CD4^+CD25^+$ T cells (Bensinger et al. 2001;Jordan et al. 2001;Kawahata et al. 2002). Upon selection, $CD25^+$ T cells constitute 5-10 % of peripheral $CD4^+$ T cells and less than 1% of peripheral $CD8^+$ T cells in normal naïve mice (Sakaguchi et al. 1995). Several other markers are expressed by the $CD4^+CD25^+$ T cells. One of the most important markers is the forkhead transcription factor FOXP3 (Fontenot et al. 2003;Khattri et al. 2003). Gene expression analysis also showed that $CD25^+$ T cells, but not $CD25^-$ cells express high levels of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Gavin et al. 2002) and glucocorticoid-induced TNFR-related protein (GITR) (McHugh et al. 2002).

In terms of the suppressive function of the natural Treg cells it has been shown that they require TCR stimulation in order to exert suppression and the antigen concentration can be 10- to 100- fold lower than that required to activate CD4+CD25- T cells (Takahashi et al. 1998). Additionally, in vitro suppression by natural Treg cells requires direct cell contact with responding T cells and/or APCs and is cytokine independent (Bluestone and Abbas 2003;Sakaguchi 2004;Thornton and Shevach 1998).

Apart from the $CD4^+CD25^+$ T cells, other $CD4^+$ subpopulations have been described with the ability to inhibit autoimmune responses (Roncarolo and Levings 2000). These cells are usually generated from naïve $CD4^+$ T-cell

populations under certain conditions. One subset of the inducible Treg cells has been generated in vitro upon culture of naïve $CD4^+$ T cells in the presence of exogenous IL-10 (Barrat et al. 2002;Groux et al. 1997). These cells termed Tr1 and have been shown to produce high levels of IL-10 as well as TGF-β, IFN-γ and IL-5 in the presence of antigen (Cottrez et al. 2000;Levings and Roncarolo 2000). Another population of $CD4^+$ T cells that exert inhibitory activity is the Th3 cells (MacDonald 1998) that can be generated upon antigen administration via the oral or intranasal route (MacDonald 1998;Weiner 2001). This populaion is characterized by high levels of TGF-β secretion (Chen et al. 1994;Faria and Weiner 1999) and has been shown to suppress autoimmune responses (Chen et al. 1994). Both Tr1 and Th3 cell populations are characterized by a cytokine-production profile rather than the expression of cell-surface markers. In contrast to CD4+CD25+ T cells, inducible regulatory cells mediate suppression through a cytokine-dependent mechanism since their suppression function can be reversed in the presence of anti-IL-10 and/or anti-TGF-β (Kitani et al. 2000;Kitani et al. 2003).

1.10.1 *Dendritic cells and regulatory T cells (Treg)*

As mentioned above, DCs play an important role in tolerance induction through the induction of regulatory T cells. Although the exact DC profile that is

required to activate or expand the Treg cells has not been determined, several lines of evidence support that the maturation stage of DCs is distinct from those that participate in the activation of Th1 and Th2 cells (Lutz and Schuler 2002;Mahnke et al. 2003a).

Some studies have suggested that immature DCs (iDCs) control the differentiation of naïve T cells into Treg cells (Akbari et al. 2001;Jonuleit et al. 2000;Mahnke et al. 2003b;Wakkach et al. 2003). Jonuleit and colleagues demonstrated that repetitive stimulation of human naïve $CD4^+$ T cells with allogeneic immature DCs results in the generation of alloantigen-specific Treg cells (Jonuleit et al. 2000). Those Treg cells secreted high levels of IL-10 and were able to inhibit antigen-specific proliferation and cytokine production by Th1 cells (Jonuleit et al. 2000). The induction of Treg by immature DCs, was further supported by in vivo administration of immature DCs pulsed with influenza matrix peptide (MP) into healthy individuals. It was found that, $CD8^+$ T cells isolated from the DC-immunized individuals 7 days later could inhibit antigen specific proliferation of T cells isolated before injection (Dhodapkar et al. 2001;Dhodapkar and Steinman 2002). Although, iDCs show phenotypic similarities with the IL-10-modulating DCs that induce anergic T cells (see 1.10 paragraph), the induction of Treg cells requires repetitive stimulation by iDCs.

In another study, a subpopulation of DCs has been described as tolerogenic and termed "semi-mature" DCs (Lutz and Schuler 2002). Addition of TNF-α in DC cultures has been shown to enhance DC maturation as assessed by increased expression of MHC class II and costimulatory molecules (Lardon et al. 1997;Yamaguchi et al. 1997) but functionally were different from DCs matured in the presence of LPS (Menges et al. 2002). Specifically, BM-derived DCs that have been cultured in vitro in the presence of TNF-α but not LPS were able to suppress EAE in an antigen specific fashion (Menges et al. 2002). It was initially shown that TNF-α treated DCs were mature in terms of the MHC class II and B7.1/2 expression but the same cells were not secreting pro-inflammatory cytokines, such as IL-12 and TNF-α, essential for efficient activation of naïve T cells. When MOG-peptide pulsed-DCs were injected into susceptible mice, inhibition of MOG-induced EAE was observed. A population of IL-10-secreting $CD4^+$ T cells was found in the spleens of MOG/DC-treated mice but not in mice receiving OVA/DCs (Menges et al. 2002). This indicates the Ag-specific induction of a Treg cell population with the ability to down-regulate autoimmune responses (see below).

A recent study provides evidence for the capacity of antigen-bearing DCs to expand $CD4^+CD25^+$ Treg both in vivo and in vitro (Yamazaki et al. 2003). More importantly, it was shown that not only mature but also immature DC

populations were able to expand the Treg cells. Expansion of $CD4^+CD25^+$ cells requires DC-T cell contact and the presence of co-stimulation through the B7 molecules. When steady state or mature DCs were adoptively transferred in vivo along with the $CD4^+CD25^+$ T cells, a robust antigen-specific proliferation of the later subset occured (Yamazaki et al. 2003). Although in most studies mature DCs (as well as immature DCs) induce the expansion of this particular Treg subset, it is still unclear what determines when mature DCs will activate $CD4^+CD25^-$ T cells (effector cells) or the $CD4^+CD25^+$ T reg subset.

In the field of EAT, it was recently shown that susceptible mice immunized with Tg in CFA and also injected with GM-SCF (induces iDCs) for 5 consecutive days did not develop thyroidits. In contrast, mice immunized similarly with Tg but received fms-like tyrosine kinase receptor 3 ligand (Flt3-L; promotes maturation of DCs) instead of GM-CSF, developed severe EAT (Vasu et al. 2003). The authors observed an increase of the $CD4^+CD25^+$ population in spleens of mice treated with GM-CSF and not with Flt3-L. Since this T cell sub-population was able to suppress the proliferation of mTg-specific T cells in vitro they hypothesized that GM-CSF treatment generates a DC population capable of expanding Treg cells (Vasu et al. 2003). However, no direct evidence was provided for the participation of specialized subset of the DCs in the proliferation of the $CD4^+CD25^+$ T cells after GM-CSF injection. In a follow up

study by the same group, adoptive transfer of $CD4^+CD25^+$ T cells isolated from GM-CSF-treated mice into mTg-challenged mice reduced the proliferation of mTg-specific T cell responses (Gangi et al. 2005). It was also shown that IL-10 secreted by $CD4^+CD25^+$ T cells was critical for the suppression of the effector cells, since administration of IL-10 mAb in vivo reversed the suppressive function of the $CD4^+CD25^+$ T cells (Gangi et al. 2005). In another study, in vivo depletion of $CD25^+$ T cells abrogatedartificially induced tolerance to mTg (Morris et al. 2003). Mice treated with anti-CD25 mAb developed significantly higher degree of thyroid infiltration than mice treated with isotype control mAb (Morris et al. 2003). Additionally, CD4+CD25+ T cells isolated from mTg-tolerized mice had the ability to suppress the proliferation of mTg-specific T cells. These data indicate the important role of CD4+CD25+ T cells in the immunoregulation of EAT.

In conclusion, there is strong evidence that DCs play a role in peripheral tolerance. Therefore, further understanding of the factors that generate DCs with the ability to induce antigen-specific tolerance will be useful in designing therapeutic methods for autoimmune diseases.

2 CHAPTER 2

2.1 MATERIALS AND METHODS

2.2 Animals

Female CBA/J (H-2^k), C3H/HeJ (H-2^k), B10.BR (H-2^k), AKR/J (H-2^k), C57BL/6 (H-2^b), DBA/1J (H-2^q), DBA/2J (H-2^d), and SJL/J (H-2^s) mice were purchased from the Jackson Laboratories (Bar Harbor, ME USA), and used in experiments between 6 to 8 weeks of age. All experimental procedures were reviewed and approved by the institutional animal care committee.

2.3 Antigens and synthetic peptides

Tg was extracted from thyroids of outbred ICR mice (Bioproducts for Science, Indianapolis, IN) as previously described (Chronopoulou and Carayanniotis 1992). In detail, frozen glands were homogenized in phosphate buffered saline (pH 7.0) and the supernatant was centrifuged three times at 16,000 x g. The clear supernatant was passed through a Sepharose CL-4B column (Pharmacia, Quebec, Canada). The fractions of peak II (Figure 2.1), were pooled, dialyzed in 1 X PBS, concentrated in ultra-filtration cells (Amicon, Danvers, MA), filter-sterilized, and stored at –20 °C at a final concentration of 2-3 mg/ml.

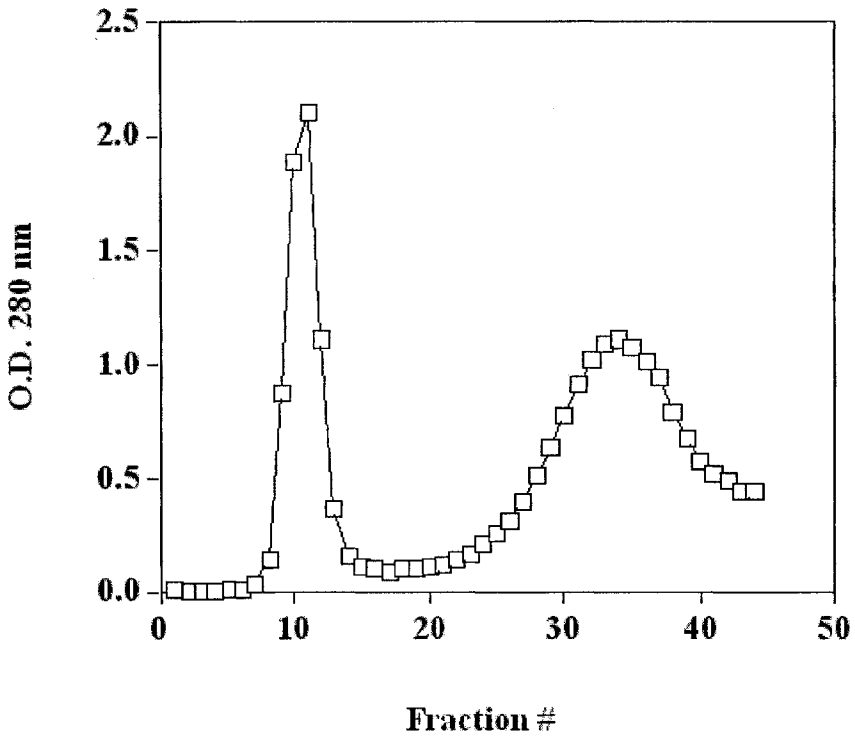


Figure 2.1 Tg isolation from thyroid glands

About 100 thyroid glands were homogenized in 5 ml ice-cold PBS. The homogenate was centrifuged 3x at 16,000g for 10 min at 4°C. The supernatant was then loaded on Sepharose CL-4B column. Tg was eluted with PBS. Fractions (~ 4 ml/tube) were collected after sample loading. Fractions from 24 to 40 within peak II were pooled and concentrated. The Tg preparation was filter sterilized and stored at -20°C.

Ovalbumin (OVA) was purchased from Sigma (St. Louis. MO, USA). Tuberculin purified protein derivative (PPD, Seruminstitut, Copenhagen, Denmark) was purchased from Cedarlane (Ontario, Canada).

Peptides designated p110 (VQCDLQRVQ), p226 (LAETGLELLLDEIY), p306 (YQTVQCQTEGMCWCV), p1579 (LVQCLTDCANDEA), p1826 (GDMATELFSP), p2026 (GSEDTEVHTYP), p2102 (SMAQDFCLQQCSRHQ) and p2596 (YGHGSLELLADVQ) were synthesized by Sigma-Genosys (The Woodlands, TX). The Tg peptide p2495 (GLINRAKAVKQFEESQG) and the truncated Tg peptide p2496 (LINRAKAVK) were synthesized at the Alberta Peptide Institute (Edmonton, Alberta, Canada). All peptides were blocked with an acetyl group at the N-terminal and an amide group at the C-terminal end. The thiol group of internal Cys residues was blocked by acetamide. Mass spectrometry and HPLC analysis were performed on each peptide to verify composition and confirm >80% purity.

2.4 Algorithm-based search for A^k-binding peptides in Tg

A computerized algorithm that predicts peptides with A^k-binding potential was described by Altuvia et al. (Altuvia et al. 1994). Two motifs were used to scan the mTg molecule for peptides that may bind to A^k molecule (Table 2.1). The "Pole Bio-Informatique Lyonnais: Network Protein Sequence Analysis" website (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattin

prot.html) was used to scan the entire Tg molecule. Among the sequences that were found to fit motif A and B we chose for our study only those in which motifs completely overlapped.

Table 2.1 I-A^k-binding motifs A and B (Altuvia et al. 1994)

	Position #	Characteristic of a.a.	[Includes]/{Excludes} a.a.
Motif A	1	Hydrogen acceptor, non-hydrophobic, not small, not aliphatic	[DEHNQ]
	2	Not an amide	{NQ}
	3	Any amino acid	[Any a.a.]
	4	Medium-sized, aliphatic, hydrophobic, no charge, not aromatic or amide	[ILTV]
	5	Any amino acid	[Any a.a.]
	6	Not aromatic	{FHWY}
	7	Hydrophobic, no charge, not an amide	[ACFILMPTVWY]
	[DEHNQ]- {NQ}-X-[ILTV]-X-{FHWY}-[ACFILMPTVWY]		
Motif B	1	Hydrogen acceptor, not small, polar, not aliphatic	[CDEHNQY]
	2	Not negatively charged	{DE}
	3	Any amino acid	[Any a.a.]
	4	Medium-sized, aliphatic, hydrophobic, no charge, not aromatic or an amide	[ILTV]
	5	Hydrogen acceptor, non-hydrophobic, not small, not aliphatic	[DEHQN]
	[CDEHNQY]- {DE}-X-[ILTV]- [DEHQN]		

2.5 Culture Media and Cell Lines

All tissue culture media including Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 (GibcoBRL, Burlington, ON, Canada) were supplemented with 10% heat-inactivated (1 hour at 56°C with moderate stirring) fetal bovine serum (FBS) (Cansera, ON, Canada), 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 5×10^{-5} M 2-ME (Sigma Chemical Co., St Louis, MO). DMEM was also supplemented with 20mM HEPES buffer (Gibco).

The BW5147$\alpha^-\beta^-$ variant (White et al. 1989) was a kind gift of Dr. P. Marrack (National Jewish Centre, Denver, CO, USA). The antigen-presenting cell line LS 102.9, a B-cell hybridoma expressing H-2A$^{d/s}$ and H-2E^d (Kappler et al. 1982), and the IL-2-dependent CTLL-2 cell line (Gillis and Smith 1977) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The HB 65 (H16-L10-4R5) clone secreting IgG2a MAb specific for nucleoprotein of influenza type A virus (Yewdell et al. 1981), the TIB 92 (10-3.6.2) clone secreting IgG2α MAb, specific for I-A^k molecules (Oi et al. 1978) as well as the HB 32 (14-4-4S) clone secreting an IgG2α MAb that specifically reacts with I-E^k determinants (Ozato et al. 1980) were also purchased from ATCC.

The X63Ag8 cell line, secreting mGM-CSF (Zal et al. 1994) was a kind gift from Dr. B. Stockinger (Department of Molecular Immunology, National Institute for Medical Research, London, United Kingdom).

2.6 EAT induction

2.6.1 *Immunization*

Mice were immunized subcutaneously (s.c.) under ether anesthesia at 2 sites (base of the tail and along the back) with 100 nmol of Tg peptide in 100 μl of 1:1 PBS:CFA (with Mycobacterium butyricum, Difco Laboratories Inc., Detroit MI, USA). Three weeks later, they were boosted s.c. with 50 nmol of Tg peptide in Incomplete Freund's Adjuvant (IFA) (Difco). Five weeks from the initial challenge mice were bled to obtain sera for ELISA assays and the thyroid were removed and fixed in 10% buffered formalin for histological examination. In some experiments EAT was induced by immunizing animals s.c. with 100 μg Tg in CFA once. Thyroids were collected 21 days from Tg injection.

2.6.2 *Adoptive transfer*

Adoptive transfer of peptide-primed LNCs were performed as previously described (Rao et al. 1999). Briefly, CBA/J mice were immunized s.c. with 100

nmol of Tg peptide in CFA, and 9 days later, draining LN were collected and single cells suspensions were prepared. Cells were cultured in the presence of the immunizing peptide (25 μM). Three days later, the cells were washed 3x with PBS and transferred intraperitoneally (i.p.) into naïve syngeneic recipients at 2 x 10^7 cells/animal. Fourteen days after transfer, mice were sacrificed and their thyroid glands were collected in 10% buffered formalin for histological examination.

2.6.3 *Evaluation of thyroid histology*

Fixed thyroid glands were embedded in methacrylate and sectioned serially (approximately 21 sections were obtained/gland). Thyroid sections were stained with hematoxylin and eosin. The mononuclear cell infiltration index (I.I.) was scored as follows: 0 = no infiltration, 1 = interstitial accumulation of cells between two or three follicles, 2 = one or two foci of cells at least the size of one follicle, 3 = extensive infiltration 10-40% of total area, 4 = extensive infiltration 40-80% of total area, and 5 = extensive infiltration >80% of total area.

2.7 Proliferation assays

2.7.1 *LNC proliferation assay*

Mice were immunized s.c. with peptide (100 nmol) in CFA as described above, and 9-10 days later, draining LN were collected. A single cell suspension was prepared and cells were centrifuged, washed and adjusted at 4 x 10^6 cells/ml in complete medium (DMEM + 10% FCS). The cells (100µl) were then cultured in the presence or absence of antigen in flat-bottomed 96-well plates (total final volume 200 µl) for 72 h at 37 °C in a 10% CO_2 90% air-humidified incubator. Eighteen hours before harvesting, 1 µCi of [^{3}H]-thymidine (6.7 Ci/mmol, DuPont, Mississauga, ON, Canada) was added to each well in 25 µl of complete medium. The cells were harvested using a Harvester 96® Mach III M (Tomtec, Hamden, CT, USA) and incorporated radioactivity was measured using the TopCount NXT™ microplate counter (Canberra Packard Canada, Mississauga, ON, Canada). Stimulation index (S.I.) is defined as (cpm in the presence of antigen/cpm in the absence of antigen).

2.7.2 *CTLL-2 proliferation assay*

In flat-bottomed wells of microtiter plates, 10^5 hybridoma T cells and an equal number of antigen presenting cells were cultured for 24 h with or without

antigen in total volume of 200 µl/well. Then 100 µl of supernatant was harvested from each well, transferred into a new plate and kept frozen for longer than 2 h at -70°C. Upon subsequent thawing, 10^4 CTLL-2 cells were added per well and 18 h later, 1 µCi of [^{3}H]-thymidine (6.7 Ci/mmol, DuPont) was added to each well in 25 µl of complete medium. The cells were harvested 6 h later and incorporated thymidine was measured, as described above.

2.8 Enzyme-linked immunosorbent assay (ELISA).

2.8.1 *Detection of IgG antibody.*

Polyvinyl chloride microwell plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with 0.2 µg of peptide or 1 µg of mTg in 100µl of carbonate buffer pH 9.6, and were incubated overnight at 4°C. The plates were washed 3x with 200 µl of PBS and then blocked overnight at 4 °C with PBS containing 1% BSA. The serum samples were added to the well (100 µl/well), were serially diluted and incubated for 1 h at room temperature. The wells were then washed 3x with 200 µl of PBS-Tween (PBS-T) and 100 µl/well of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) diluted in 1% BSA PBS-T (1:2000) was added. After one hour incubation at room temperature, the plates were washed 3x with PBS-T followed by the addition of 100 µl of p-

nitrophenylphosphate substrate (Sigma) solution (1mg/ml substrate in 10 % diethanolamine, pH 9.8, 100 µl/well) and were incubated for 1 h at room temperature. Absorbance of the p-nitrophenylphosphate product was measured at 405 nm using an automated microplate reader (Molecular Devices, Sunnydale, CA, USA).

2.8.2 *Detection of cytokines in culture supernatants.*

Cytokine production was determined in culture supernatants harvested following 48 h stimulation of antigen-primed LNCs with the respective antigen (8-20 µM). Detection of IL-2, IL-4, IL-10 and IFN-γ was performed by sandwich ELISA based on non-competing pairs of capture and detection (biotinylated) mAbs as follows: IL-2, JES6-1A12 and JES6-5H4; IFN-γ, R4-6A2 and XMG1.2 (all from BD PharMingen, San Diego, CA); IL-4, 11B11 (ATCC) and DVD-6-24G2 (BD PharMingen). IL-10 was detected via the use of affinity purified polyclonal rabbit Ab 500-P60 and 500-P60Bt (PeproTech, Inc., Rocky Hill, NJ, USA). In brief, 96-well polyvinyl chloride microwell plates (Dynatech Laboratories) were coated overnight with 1 µg/ml of the IL-2, IL-4 and IL-10 capture mAb and 4 µg/ml for the IFN-γ capture mAb. The wells were then washed and blocked with 1% BSA for 1 h at room temperature. Both cytokine standards and the LNC supernatants were then added (100 µl/well) and the plates were incubated at 4 °C overnight.

The biotinylated anti-cytokine secondary Ab was added in the wells (100 μl/well) at a concentration of 2 μg/ml for IL-2 and IL-10 and 1 μg/ml for IL-4 and IFN-γ. Following one hour incubation at room temperature, alkaline phosphatase-conjugated streptavidin and its substrate (Sigma) were added as described above. Absorbance at 405 nm was measured by using a microplate reader (Molecular Devises). The amount of cytokine in each supernatant was extrapolated from the standard curve for the respective cytokine. Standard curves were generated for each individual cytokine using known amounts of murine rIL-2 and rIFN-γ (BD PharMingen), or rIL-4 and rIL-10 (PeproTech). The detection limits were 4 pg/ml for IL-2 and IFN-γ, 10 pg/ml for IL-4, and 17 pg/ml for IL-10.

In some experiments, detection of IL-2, IL-4, IL-10, IL-12p70, IL-1β, IL-6 and IFN-γ in culture supernatants, was performed by sandwich ELISA using the BD OptEIA™ mouse ELISA set (BD Biosciences), following the manufacturer's recommendations. TGF-β1 was measured by the TGF-β Emax Immuno Assay System (Promega, Madison WI). Light absorbance at 450 nm was measured using a Vmax plate reader (Molecular Devices, Sunnyvale, CA).

2.9 Fluorescence-activated cell sorting (FACS) analysis

Expression of the Vβ4 chain gene on the surface of the 9.13 T-cell hybridoma was determined by Fluorescence Activated Cell Sorting (FACS) analysis. Cells grown in log-phase were washed once and re-suspended in PBS buffer containing 1% BSA and 0.1 % NaN_3 (FACS buffer) and finally were adjusted to 10^7 cells/ml. The BW5147 $\alpha^-\beta^-$ cell line that was utilized for the generation of the 9.13 T-cell clone, was used as control. 10^7 cells/ml from both cell lines (10^6 cells per 100 μl) was incubated with 10 μg/ml of the I-A^s–specific HB4 mAb (IgG2b) (ATCC). Following 30 min incubation on ice, the cells were washed 3x with FACS buffer and were labeled with 1 μg of anti-mouse CD4 mAb labeled with fluorescein isothiocyanate (FITC) and 1 μg of anti-mouse Vβ4 TCR mAb or 1μg of anti mouse αβ TCR mAb both coupled with R-phycoerythrin (R-PE) (all from PharMingen). Cells were subsequently incubated at 4°C in the dark for 30 minutes, were washed 3x with FACS buffer, and were suspended in 500 μl of 1x PBS buffer containing 1% paraformaldehyde (Sigma Chemicals). The fluorescence of 10^4 cells was measured by FACStar Plus analyzer and the results were analyzed using Cellquest software (Becton-Dickinson Inc., Franklin Lakes, NJ, USA).

To determine the phenotype of DCs, FACS analysis was performed as described above. PE-labeled hamster anti-CD11c (clone HL3), and rat anti-I-A^k

(clone 10-3.6), hamster anti-CD80 (clone 16-10A1), rat anti-CD86 (clone GL1), rat anti-CD11b (clone M1/70), rat anti-CD40 (clone 3/23), rat anti-CD45R (clone RA3-6B2), rat anti-CD8a (clone 53-6.7), all labelled with FITC, were purchased from PharMingen.

The surface phenotype of the $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell subsets was characterized by using the following antibodies: rat anti-CD103 (clone M290), hamster anti-CD69 (clone H1.2F3), rat anti-CD137 (clone 1AH2), and rat anti-CD62L (clone MEL-14), all labelled with FITC (PharMingen). Appropriate isotype-matched PE-or FITC-conjugated mAbs were purchased from PharMingen. Rat anti-GITR (clone# 108619) and isotype control both FITC-labeled were purchased from R&D Systems (Minneapolis, MN). Purified hamster anti-CD152 (clone 9H10) and isotype-matched FITC-labeled mAb were purchased for Pharmingen.

2.10 Generation of bone marrow-derived dendritic cells (BM-DC).

Dendritic cells were generated from bone marrow (BM) progenitors as previously described (Lutz et al. 1999). Briefly, BM was prepared from femurs and tibias of CBA/J mice, and RBC cells were lysed with NH_4Cl. For cell culture, sterile petri dishes with 100 mm diameter (Corning Incorporated, Corning, NY USA) were used. At day 0, BM cells were seeded at 2×10^6 per 100 mm dish in 10 ml RPMI 1640 medium supplemented with 10 % GM-CSF supernatant. At day 3,

another 10 ml of media containing 10 % GM-CSF were added to the plates. At days 6 and 8, half of the culture supernatant was collected, centrifuged and the cells were resuspended in 10 ml fresh medium containing 10 % GM-CSF supernatant, and given back into the original plate. At day 10, non-adherent cells were collected and used for further study. Semi-mature DCs (sm-DCs) or mature DCs (m-DCs) were generated by the addition of 40 ng/ml tumor necrosis factor α (TNF-α, Peprotech) or 1 μg/ml lipopolysaccharide (LPS, Sigma) at day 9 of culture. Twenty-four hours later non-adherent cells were gently dislodged and harvested for further study.

2.11 Adoptive transfer of sm-DCs and EAT induction.

On day 9 of DCs generation, cells were pulsed with Tg (100 μg/ml) or OVA (100 μg/ml) for 6h and were subsequently treated with TNF-α (40 ng/ml) for 24h. TNF-α-treated DCs (sm-DCs) were then washed with PBS and adjusted at 2.5×10^7 cells/ml. Cells were intravenously (i.v.) injected into CBA/J mice (100 μl/mouse) at days 1, 3 and 5. As a control, a third group of mice received PBS i.v. on days 1, 3, and 5. On day 7 from the initial injection, all mice were immunized with Tg (100 μg/mouse) in CFA, and 21 days later, thyroid glands were removed to assess pathology. Spleens were also collected for further study.

2.12 Isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells.

Spleen tissues from CBA/J mice were passed through a sterile stainless steel mesh (Sigma), and single cell suspensions were prepared. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, were purified using a magnetic cells sorting (MACS) $CD4^+CD25^+$ regulatory T cell isolation kit (mouse) according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA). Briefly, $CD4^+$ T cells were isolated by depletion of magnetically labeled non-$CD4^+$ T cells. $CD4^+$ T cells labeled with PE-conjugated anti-CD25 and were then magnetically labeled with anti-PE MicroBeads provided with the kit. The magnetically labeled cells were passed through a column placed in the magnetic field of MACS separator. The $CD4^+CD25^+$ T cells retained in the column and the flow through was shown to be >90% of $CD4^+CD25^-$ T cells. Following elution, the $CD4^+CD25^+$ T cells were shown to be >90% by flow cytometric analysis.

2.13 Mixing and transwell experiments

CBA mice were immunized subcutaneously (s.c.) with 100 μg of Tg in CFA and 9 days later, draining lymph nodes were isolated and single cell suspension was prepared. $CD4^+CD25^-$ T cells (effector cells), purified as described above, were cultured (1×10^5 cells/well) in the presence or absence of Tg (100 μg/well) in

flat-bottom 96-well plates for 4 days at 37 °C in a 10% CO_2, 90 air-humidified incubator. Splenocytes (2 x 10^5 cells/well) isolated from syngeneic naïve mice and treated for 15 min with 50 μg/ml mitomycin C (Sigma) were used as APC. Mixing experiments were performed by the addition of equal numbers (1 x 10^5 cells/well) of $CD4^+CD25^+$ T cells, isolated from DC-treated CBA mice and syngeneic $CD4^+CD25^-$ T cells. Eighteen hours before harvesting, 1μCi of [^{3}H] thymidine was added to each well in 25 μl of complete medium.

Transwell experiments were performed in 24 -well plates (0.22 μm pore size; Costar). 6 x 10^5 $CD4^+CD25^-$ effector T cells (isolated from Tg-challenged mice as described in the previous paragraph) were cultured with 1.2 x 10^6 APC in the presence of 100 μg/ml Tg and 6 x10^5 $CD4^+CD25^+$ T cells were placed in the same or in the top chamber. When $CD4^+CD25^+$ T cells cultured in the top chamber, APC and Tg were added at the same chamber. After 3 days of culture, the transwell chamber was removed and 1μCi of [^{3}H] thymidine was added to the lower well.

2.14 Suppression of Tg-induced EAT

CBA/J mice were challenged with Tg- or OVA-pulsed DCs as described above and 21 days later spleens were collected. $CD4^+CD25^+$ T cells were prepared and 5 x 10^5 cells/mouse were administered i.p. into syngeneic naïve recipients. Control mice received one i.p. injection of PBS. One day later, some mice were

immunized s.c. with 100 μg of Tg in CFA and 21 days after Tg challenge, all mice were sacrificed and thyroid glands were collected. Thyroid pathology was assessed as described above.

2.15 RNA isolation

Total RNA was extracted using the TRIzol reagent (Gibco-BRL, Burlington, ON, Canada) following the manufacturer's protocol. Briefly, $5x10^6$ cells were lysed in 1 ml of TRIzol reagent. Addition of 200 μl chloroform (Sigma Chemicals) followed by centrifugation separates the solution into an aqueous phase, that contains the RNA, and an organic phase. The aqueous phase (600 μl) was transferred in a new diethyl pyrocarbonate-treated (DEPC) tube and RNA recovered by precipitation with isopropyl alcohol (500 μl). The isolated RNA washed with 70% ethanol (1 ml), air-dried and resuspended in 20 μl of RNAase-free water (Sigma Chemicals). The RNA yield as well as its purity was measured using a spectrophotometer at the optical densities of 260 and 280 nm.

2.16 First-strand cDNA synthesis and Genomic DNA extraction

First-strand cDNA synthesis was performed using a kit from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden). Briefly, 5 μg of total RNA was heated

in 65°C for 10 min to remove any RNA secondary structure, and then chilled on ice. The RNA was then added in a mixture containing 11 μl first-strand bulk mix, 1μl dithiothreitol (DTT) solution and 0.2 μg (1 μl) Not I-d(T)$_{18}$ primer, all provided with the kit. The enzyme that catalyzed the reaction contained in the First-strand Bulk mix is Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase. The mixture was then incubated for 1 hour at 37°C and the resultant product was heated at 75 °C for 10 min to terminate the reaction. The cDNA was stored at -20°C.

Genomic DNA was extracted from 5 x 10^6 cells by using the "DNeasy Tissue Kit" (Qiagen) and following the manufacturer's instructions.

2.17 Polymerase Chain Reaction (PCR)for gene amplification

First strand cDNA was amplified by PCR in a reaction mixture containing 1x PCR buffer, 2 mM $MgCl_2$, (both from Invitrogen, Carlsbad, CA, USA), 0.2 mM of each dNTP (Gibco-BRL), 20 pmoles of each primer (forward and reverse, Operon Technologies, Inc. Alameda, CA, USA), 1 μl of cDNA and 2.5 U of Taq polymerase (Invitrogen) in 100 μl final volume of RNAase-free water. A 35-cycle step program was conducted using the Perkin Elmer DNA thermocycler (Cetus, Norwalk, CT, USA) with 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C preceded by a 5 min denaturation step at 94 °C. The

amplified PCR product was subjected to agarose gel electrophoresis containing ethidium bromide (0.5 μg/ml) for product visualization.

2.18 RNA ligase-mediated rapid amplification of 5′ cDNA ends (RLM-RACE)

The GeneRacer Kit (Invitrogen) was used in order to identify the 5′ end of the TCR α chain gene of a Tg peptide-specific T cell hybridoma. Briefly, total RNA was extracted from the 9.13 T-cell hybridoma as described above and was treated with calf intestinal phospatase (CIP – provided with the kit) to remove the 5′ phosphates, followed by treatment with tobacco acid pyrophosphatase (TAP- – provided with the kit) to remove the 5′ cap structure from full-length mRNA. The GeneRacer RNA Oligo (provided with the Kit) was ligated next to the 5′ end of the mRNA using T4 RNA ligase. The mRNA was then reverse-transcribed to cDNA using an oligo dT primer (supplied with the kit). Amplification of cDNA was performed with an oligo-specific forward primer (GeneRacer™ 5′ primer Table 2.2) and a reverse primer, specific for the α constant chain (Cα-R Table 2.2). Material from the first round PCR was subjected to nested PCR using, as forward primer a nested oligonucleotide of the RNA oligo (GeneRacer™ 5′ nested primer Table 2.2) and as reverse primer, a nested Cα oligonucleotide (either Cα1-R or Cα2-R Table 2.2). The amplified products were subjected to agarose gel electrophoresis, and the band containing the α

chain gene was cut and purified using the QIAquick Gel Extraction Kit (Qiagen) in order to be cloned.

2.19 Cloning of the 9.13 TCR α chain gene

In order to sequence the α chain segment, the amplified PCR product was gel extracted and cloned into the TOPO TA cloning kit (Invitrogen). According to this method, Topoisomerase I from Vaccinia virus is covalently bound to a plasmid vector (pCR®4-TOPO), which is supplied linearized. The enzyme binds to double-stranded DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand, generating single, overhanging 3′ deoxythymidine (T) residues. Taq polymerase has a nontemplate-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3′ ends of PCR products. This allows the PCR inserts to ligate efficiently with the vector. TOPO cloning reaction was performed for 5 min at room temperature, and 2 μl of the mixture were transferred to a vial containing TOP10 One Shot Chemically Competent E.coli (Invitrogen). Upon incubation on ice for 30 min, the cells were heat-shocked for 30 seconds at 42 °C, and transferred immediately to ice where 250 μl of SOC medium (Invitrogen) were added. After 1 h incubation at 37 °C with moderate shaking (200 rpm), 100 μl from the reaction were spread on a pre-warmed Luria Bertani (LB) agar plate containing 100 μg/ml of ampicillin

(Sigma Chemicals, St. Louis, MO, USA) and incubated for 12-16 h at 37 °C. Plasmid DNA purification was performed with the plasmid mini-prep DNA extraction kit (Mississauga, ON, Canada). Purified plasmid DNA was then subjected to PCR amplification by using a forward vector-specific primer (T7-F, Table 2.2) and the Cα-R2 reverse primer. Positive plasmids were sequenced in order to identify the entire gene sequence.

2.20 Amplification of the 9.13 TCR α chain gene segments from genomic DNA.

Genomic DNA that was extracted from the 9.13 T-cell hybridoma, was used as template in order to amplify the Leader(L)-Variable(V)-Joining(J) segment of the TCR α chain by PCR. The amplified segment was ligated into the pTα cassette vector kindly provided by Dr. D Mathis (Joslin Diabetes Center Harvard Medical School, MA, USA). Two forward primer sequences, that contain an overlapping region, were designed to engineer a unique XmaI restriction site 20 bp 5′ of the ATG starting codon and a pTα vector-specific sequence. The first primer amplifies the beginning of the leader Vα1.1 sequence (LVα1.1-F, Table 2.2), and the second primer, contains an overlapping sequence with the first primer and also introduces the XmaI restriction site (XmaI-pTα-F, Table 2.2). A sequence located 30 bp within the intronic region downstream of Jα49 segment (EMBL/GenBank data libraries accession number 15795314) was used to design

reverse primer. The oligonucleotide was designed to introduce the Not-I restriction site for subsequent cloning (Jα49-inton-NotI-R Table 2.2).

The length of the intronic region between the L and the V region of the gene was calculated based on the published genomic sequence of the mouse TCR α locus (EMBL/GenBank data libraries accession number 15795314). Resolution of the PCR reaction product by 1.5% agarose gel was performed and the band contained the LVJα gene was gel-extracted, purified and cloned into pCR4-TOPO vector as described above. TOP 10 E. coli competent cells were transformed with the cloned product and plasmid DNA was purified and sequenced.

2.21 Cloning of the 9.13.TCR β chain gene.

The variable region of the TCR β chain expressed by the 9.13 T cell hybridoma belongs to Vβ4 family (Rao et al. 1997). In order to amplify and clone the entire β chain gene, a forward primer specific for the beginning of the leader Vβ4 gene sequence (KpnI-Vβ4-F, Table 2.2) and a reverse primer specific for the 3′ of the Cβ region (XbaI-Cβ1-R Table 2.2) were used. The primers were designed to introduce the unique Kpn-I and Xba-I restriction sites, in order to facilitate subsequent cloning. Upon amplification, the PCR product was subjected in agarose gel (1.5%) electrophoresis and the expected band was cut and purified using the QIAquick Gel Extraction Kit (Qiagen).

The purified β chain gene was digested with KpnI and XbaI restriction enzymes (Promega, Madison, MI, USA). Briefly, 5 μl of the PCR product were mixed with 1 μl of each restriction enzyme (10 U/μl each), 0.5 μl of BSA Acetylated (1 mg/ml) and 5 μl of MULTI-CORE buffer 10X (all from Promega) in a total volume of 20 μl of nuclease-free water (Sigma). After incubation for 1 h at 37 °C, the digested product was gel purified and re-concentrated by ethanol precipitation for subsequent ligation reaction. The pcDNA3.1/zeo$^+$ mammalian expression vector (Invitrogen) was similarly digested, gel purified and mixed with the DNA insert in two different vector: insert (1:1 and 1:2) ratios, in order to identify the optimum ratio for efficient ligation. The ligation reaction was performed in the presence of T4 DNA ligase (Invitrogen) for 30 min at room temperature. TOP10 One Shot Chemically Competent E.coli were transformed as described above and 100 μl from the reaction were spread on a pre-warmed LB agar plate containing 100 μg/ml of ampicillin and incubated for 12-16 h at 37 °C. Positive colonies were identified by PCR with a vector specific forward primer (from the T7 promoter priming site) and an insert specific reverse primer (the Cβ1-R primer). DNA plasmids were purified with the plasmid mini-prep DNA extraction kit, and those that were found to contain the β chain gene were sequenced in order to identify the entire β chain sequence.

2.22 Amplification of the 9.13 TCR β chain gene segment from genomic DNA

The L-V-Diversity(D)-J β chain segment was amplified from genomic DNA of the 9.13 T cell hybridoma by PCR for ligation into the pTβ cassette vector (also provided by Dr. D. Mathis). Primers were designed to introduce a unique XhoI restriction site 7 bp 5′ of the starting codon (XhoI-pTβ-F, Table 2.2), and a novel ApaI-SacII 35 bp 3′ within the intronic sequence of Jβ2.1, (Jβ2.1-ApaI-SacII-R, Table 2.2). The length of the intronic region between the L and the V region of the gene was calculated based on the published genomic sequence of the mouse TCR β locus (EMBL/GenBank data libraries accession number 2358100). The PCR product was visualized in 1.5% agarose gel, and the respective β chain gene was cut and gel purified as described above. To ensure fidelity during amplification, the β chain gene was digested with XhoI and ApaI and was cloned into the multi-cloning site of the pcDNA3.1/zeo+ mammalian vector, which had previously digested with the same restriction enzymes. Upon transformation of TOP 10 E. coli competent cells, several colonies were identified. Colonies were tested by PCR amplification for the presence of the insert. For this reason a vector specific primer (T7, same as above) and a gene specific primer (Jβ2.1 -R, same as above, for the β chain) were used. DNA plasmids were extracted from positive colonies as described above and were sequenced.

2.23 Preparation of the pTα and pTβ constructs that contain the 9.13 TCR α and β chains

To generate the constructs that contain the α and β chain of the 9.13 T cell hybridoma, we used the pTα and pTβ cassette vectors (Kouskoff et al. 1995), kindly provided by Dr. D. Mathis. To confirm the correct profile of the cassette vectors several restriction enzyme digestions were performed. The following restriction enzymes were used: BamHI, SalI and SalI/NotI for pTα and EcoRI, KpnI and XhoI/SacII for pTβ (all from Promega) and the digestion reactions were performed according to the manufacturer's protocol.

Sequenced α and β chain segments that were found to be in frame upon comparison with the genomic sequences, were excised from the pCR4-TOPO and pcDNA3.1/zeo$^+$ vectors respectively. Excision of the TCR α chain gene was performed with the enzymes XmaI and NotI, and TCR β chain gene with the XhoI and SacII restriction enzymes. The resulting products were quantified using the 2-Log DNA Ladder (New England Biolabs Beverly, MA, USA). The TCRα and TCRβ expression cassette vectors were also digested with Xma-I/Not-I and Xho-I/Sac-II enzymes respectively and were quantified using the same DNA ladder. Ligation of the LVJα and LVDJβ into the cassette vectors was performed in equimolar (1:1) ratio at room temperature for 12h. Upon transformation of XL-10 Gold Ultracompetent cells (Stratagene, La Jolla, CA, USA) with the pTα or

pTβ constucts, several colonies (~50) were obtained. DNA plasmids were purified and were screened with restriction enzyme digestion reactions to identify the vectors with the correct profile and also with PCR to verify the presence of the desired genes. For amplification of the α chain the LVα1.1-F (Table 2.2) forward primer was used in combination with a reverse primer from the unique VJα-R region (VJα-R, Table 2.2). For the β chain amplification, a forward primer within the Vβ4 sequence (Vβ4-F, Table 2.2) and a reverse primer from the unique VDJβ region (VDJβ-R, Table 2.2) were used.

2.24 Genotyping for identification of 9.13 TCR transgenic mice.

9.13 TCR transgenic mice were identified by a PCR screening strategy using primers specific for 9.13TCR α and β chain. Briefly, mice tail biopsies were performed at age of 4 weeks and genomic DNA was extracted as described in Materials and Methods. A duplex PCR which contains two pairs of primers was set up to examine the presence of 9.13 TCR transgenes. For detection of 9.13TCR α-chain-gene 5'GATTTTCCTGAATACATCTCCCG-3' and 5'-CCAAAATAGAAGTTCTGGTAACC-3', as well as PCR internal control primers which amplifies TCRd exon 1 gene, 5'-CAAATGTTGCTTGTCTGGTG-3' and 5'-GTCAGTCGAGTGCACAGTTT-3', were used. Similarly, 9.13TCR β gene-specific primers 5'-CTCACCAAAGAGACCAGTATCTC-3' and 5'-

CACACACAAACCCGCGGACT-3', in addition to PCR internal control primers, were used to identify 9.13 TCR β transgene. Approximately 100 ng DNA was added to each 50 μl PCR reaction which contains 2 mM Mg^{2+}, 0.4 μM primers, 200 mM dNTPs and 1 U Taq polymerase. Cycling was performed in a PTC-100 thermal cycler (MJ Research, Waltham, MA) with 35 cycles of 94 ºC 15s, 57 ºC 15s, 72 ºC 45s, which was followed by a extension step at 72 ºC for 5 min. In each run, a no-template control and a plasmid control were included. Final PCR products were analysed by agarose gel eletrophoresis.

Table 2.2 Primer list

Denotation	5′Sequence3′
KpnI-Vβ4-F	GCGGTACCATGGGCTCCATTTTC
XbaI-Cβ1-R	GCCGTCTAGATCAGGAATTTTTTTTC
XhoI-Vβ4-F	ACTCTCGAGAGGAAGCATGGGCTCCA
Vα1.1-F	AGGTGCAGCAGAGCCCAGAA
L-Vα1.1-F	CTCACTGCCTAGCCATGAAATCCTTG
Jα49-intron-NotI-R	CTGCGGCCGCCAGAATTCTACAG
Jβ-2.1-R	GAGGGCCCGCGGACTGTCCTACCTT
Jβ-2.1-ApaI-SacII-intron-R	CTGGGCCCGCGGTGACTGTCCTACTT
XmaI-pTα-F	GACCCGGGCTTCTCACTGCCTAG
XhoI-pTβ-F	GACTCGAGAGGAAGCATGAGCT
Cα-R	AAA TCC CGC TAC TTT CAG CA
Cα-R1	GGA ACG TCT GAA CTG GGG TA
Cα-R2	CAG TTT TGT CAG TGA TG
VJα-R	CTG GTA ACC CGT GTT AGC TGC AC
Vβ4-F	CCT CAA GTC GCT TCC AAC C
VDJβ-R	GAA GAC GCC CAG TCT TGG CTG CT
GeneRacer™ 5′ primer	CGACTGGAGCACGAGGACACTGA
GeneRacer™ 5′ nested primer	GGACACTGACATGGACTGAAGGAGTA
T7-F	TAATACGACTCACTATAGGG

3 CHAPTER 3

Delineation of five thyroglobulin T-cell epitopes with pathogenic potential in experimental autoimmune thyroiditis

3.1 Abstract

Experimental autoimmune thyroiditis (EAT) is a T-cell mediated disease that can be induced in mice following challenge with thyroglobulin (Tg) or Tg peptides. To date, five pathogenic Tg peptides have been identified, four of which are clustered toward the C-terminal end. Because susceptibility to EAT is under control of H-2A^k genes, we have used an algorithm-based approach to identify A^k-binding peptides with pathogenic potential within mouse Tg . Eight candidate synthetic peptides, varying in size from 9 to 15 a.a., were tested and five of these (p306, p1579, p1826 p2102 and p2596), were found to induce EAT in CBA/J (H-2^k) mice either after direct challenge with peptide in adjuvant or by adoptive transfer of peptide-sensitized lymph node cells (LNC) into naive hosts. These pathogenic peptides were immunogenic at the T-cell level eliciting specific LNC proliferative responses and IL-2 and/or IFN-γ secretion in recall assays in vitro but contained non dominant epitopes. All immunogenic peptides were confirmed as A^k binders since peptide-specific LNC proliferation was blocked by an A^k-specific but not a control mAb. Peptide-specific serum IgG was induced only by p2102 and p2596 but these Abs did not bind to intact mTg. This study reaffirms the predictive value of A^k-binding motifs in epitope mapping and doubles the number of known pathogenic T-cell determinants in Tg that are now found scattered throughout the length of this large autoantigen. This knowledge

may contribute toward our understanding of the pathogenesis of autoimmune thyroiditis.

3.2 Introduction

Experimental autoimmune thyroiditis (EAT) can be induced in genetically susceptible mice after challenge with thyroglobulin (Tg) in adjuvant (Champion et al. 1992;Charreire 1989;Rose et al. 1971;Weetman and McGregor 1994). A salient feature of the disease is the gradual destruction of the thyroid gland by infiltrating mononuclear cells, a process which also takes place in Hashimoto's thyroiditis in humans, leading to hypothyroidism (Weetman and McGregor 1994). There is strong evidence that $CD4^+$ T cells are both sufficient and necessary in EAT induction because the disease can be transferred into naïve recipients by $CD4^+$ T cell lines or clones (Maron et al. 1983;Romball and Weigle 1987), does not develop in nude mice (Vladutiu and Rose 1975), and is under the control of H-$2A^k$ genes in mice bearing the susceptible H-2^k haplotype (Beisel et al. 1982a;Kong et al. 1997;Vladutiu and Rose 1971).

Evidence supporting the view that Tg is a major autoantigen in clinical or experimental thyroiditis dates back to 1956 (Roitt et al. 1956;Rose and Witebsky 1956). The molecule is quite abundant in the thyroid gland representing 75-80% of the total protein in the thyroid extract (Shulman 1971), and it can be easily

isolated by gel filtration chromatography. These features, however, did not aid in the identification of pathogenic T-cell epitope sites by biochemical methods as this was hampered by the large size of the molecule (homodimeric mass = 660 kDa). In addition, previous T-cell epitope mapping efforts could not avail themselves of the mouse Tg (mTg) gene sequence information and utilized either the human (Malthiery and Lissitzky 1987), or the partial rat (Di Lauro et al. 1985) Tg sequence data in EAT studies, relying on the high sequence homology among Tg from different species. Despite these difficulties, over the last decade, and via a variety of methods, five immunopathogenic Tg peptides have been identified, encompassing at least six distinct T-cell epitopes (Carayanniotis and Rao 1997). The use of Tg peptides as model antigens in EAT generated an impetus for studying the immunoregulation of the disease (Carayanniotis and Kong 2000). At the same time, the emerged map of the pathogenic Tg epitopes focused attention on new issues that remain unresolved. First, none of the known pathogenic Tg peptides appears to comprise an immunodominant epitope, since these peptides cannot be generated following processing of intact Tg by antigen presenting cells in vitro (Carayanniotis and Kong 2000;Carayanniotis and Rao 1997). The experimental evidence, however, clearly suggests the presence of dominant A^k-binding epitopes(s) within Tg as supported by the known genetic control of the Tg-mediated EAT by the I-A^k locus (Beisel et al. 1982a;Kong et al. 1997), and the

prevention of EAT by treatment of mice with A^k-specific antibodies (Vladutiu and Steinman 1987b). Second, four out of the five known pathogenic peptides are clustered toward the C-terminal end of Tg, raising the question whether the rest of this large molecule can contain additional EAT-causing epitopes.

In this study, we proceeded to do a systematic search of the complete mTg sequence (Kim et al. 1998) for the detection of dominant and/or additional EAT-causing T-cell epitopes by utilizing an algorithm (Altuvia et al. 1994) that searches for A^k-binding motifs within a protein sequence. This algorithm takes into account the physicochemical characteristics and structural properties of amino acids within motifs that are shared among immunogenic A^k-binding peptides. The study was undertaken in the EAT-susceptible strain CBA/J ($H\text{-}2^k$) but suggests an approach that can be applied equally well to EAT-susceptible strains of other H-2 haplotypes or to other thyroid autoantigens.

3.3 Results

3.3.1 *Prediction of Tg peptides containing I-Ak- binding motifs*

The complete mTg sequence (Kim et al. 1998) was scanned for the presence of two A^k-binding motifs; a heptamer motif A and a pentamer motif B (Table 3.1), by using the algorithm described by Altuvia et al. (Altuvia et al.

1994). This computerized method was developed following a compilation of an extended database of helper T-cell sites and takes into account physical-chemical and structural properties of peptides - dictated by the primary a.a. sequence that may be responsible for binding to MHC class II antigens. Sixty nine and forty seven sites containing motif A or B, respectively, were identified (data not shown). To maximize the chances for success, we focused our attention to thirteen peptides encompassing completely overlapping motifs A and B flanked by four a.a. (Table 3.1). Of these, peptides (224-238) and (228-242) identified an overlapping motif-rich site, thus prompting the synthesis of a single peptide (226-239) containing this region. The peptides (824-838) with three proline residues flanking the motifs, and (837-851) with a proline residue inside a motif were not considered for further study, because of concerns that Pro may drastically affect the secondary structure. Peptides (2490-2504) and (2543-2557) were shown previously to contain EAT-causing T cell epitopes (Chronopoulou and Carayanniotis 1992;Rao et al. 1999). With these considerations in mind, we proceeded to synthesize the eight motif A and B -containing peptides p110, p226, p306, p1579, p1826, p2026, p2102, and p2596 (Table 3.1) and examine their immunopathogenic propepties.

Table 3.1 Putative A^k-binding peptides within the mTg sequence.

a.a coordinates[a]	Motif-containing sequence[b]	peptide synthesized	Peptide denotation
107 - 121	YAPVQCDLQRVQCWC	VQCDLQRVQ	p110
224 - 238	RELAETGLELLLDEI[c]	LAETGLELLLDEIY	p226
228 - 242	ETGLELLLDEIYDTI[c]		
306 - 320	YQTVQCQTEGMCWCV	YQTVQCQTEGMCWCV	p306
824 - 838	QDVPQVVLEGATTPP[d]	N.D.[e]	
837 - 851	PPGENIFLDPYIFWQ[d]	N.D.	
1577 - 1591	SPLVQCLTDCANDEA[f]	LVQCLTDCANDEA	p1579
1823 - 1837	DFPGDMATELFSPVD	GDMATELFSP	p1826
2025 - 2039	CGSEDTEVHTYPFGW	GSEDTEVHTYP	p2026
2102 - 2116	SMAQDFCLQQCSRHQ[f]	SMAQDFCLQQCSRHQ	p2102
2490 - 2504	PSQDDGLINRAKAVK[g]	N.D.	
2543 - 2557	YYSLEHSTDDYASFS[g]	N.D.	
2594- 2608	ESYGHGSLELLADVQ	YGHGSLELLADVQ	p2596

3.3.2 *Identification of T-cell activating peptides encompassing A^k-binding epitopes*

In order to examine the immunogenicity of the Tg peptides, CBA/J mice were challenged s.c. at two sites on the back with 100 nmol of each peptide. Nine days later, the inguinal, axillary and brachial LNC were collected and cultured in the presence of varying concentrations of the immunizing peptide. Five out of eight Tg peptides, p306, p1579, p1826, p2102 and p2596 (Figure 3.1) induced significant and specific LNC proliferation since there was no detectable response against the control peptide p2495. This reactivity profile correlated well with the capacity of the same peptides to elicit IL-2 (Figure 3.1) and/or IFN-γ (Figure 3.1) release from such activated LNC. IL-4 and IL-10 were undetectable in all culture supernatants (data not shown), indicating that these peptides – with the exception of p2102 which elicited IL-2 but not IFN-γ release – activated Th1 cells. In all cultures, peptide-specific proliferation was significantly blocked by an I-A^k-specific, but not by a control NP-specific mAb (Figure 3.2) strongly suggesting that recognition of these five immunogenic peptides occurred in the context of A^k molecules. These results confirmed the predictive value of the algorithm and the identification of five new Tg peptides encompassing A^k-restricted T-cell epitopes.

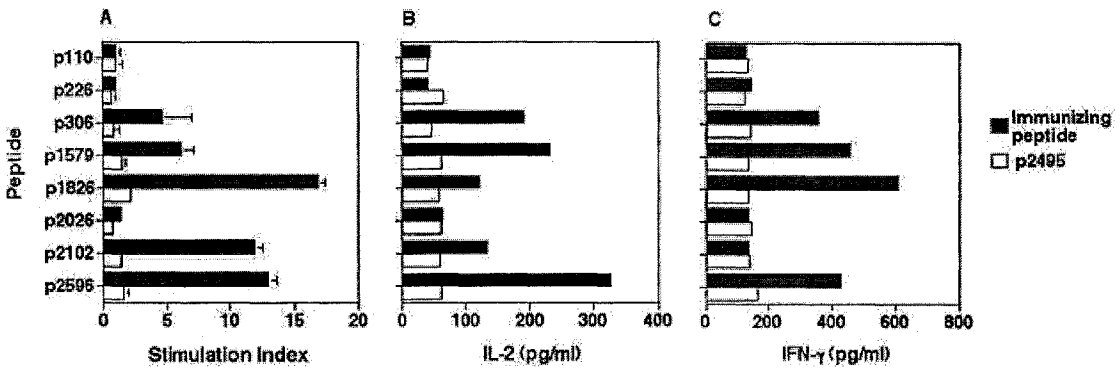


Figure 3.1 Assessment of peptide immunogenicity in CBA/J mice.

A, Recall proliferative responses of peptide-primed LNC in the presence of 25 μM of the respective peptide or p2495 as a control. Data represent the mean S.I. values of triplicate wells that were obtained from full peptide titration curves and are representative of four experiments. Background cpm ranged between 500 and 2000. B and C, Determination of cytokines by sandwich ELISA in 48h culture supernatants of peptide-primed LNCs incubated in the presence of 10-20 μM of the respective peptide or p2495 (control). IL-4 and IL-10 were undetectable (with detection limits 10 and 17 pg/ml, respectively) under similar conditions. Results are representative of two separate experiments.

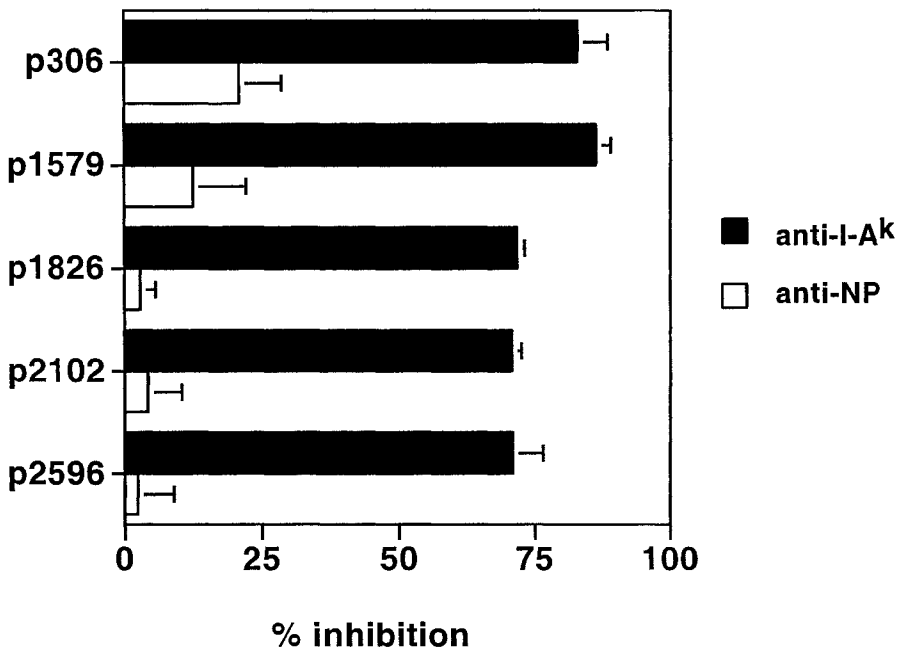


Figure 3.2 Inhibition of LNC proliferative responses against various Tg peptides.

LNC were isolated from Tg peptide-immunized CBA mice and cultured in the presence of the respective peptide (final concentration 25 μM) and mAbs (10 μg/ml) specific for I-A^k or influenza nucleoprotein (control). Antigen specific proliferation was measured by [^{3}H] uptake as described in Materials and Methods. Data represent mean +/- S.D. of triplicate wells.

3.3.3 *The immunogenic Tg peptides do not contain immunodominant determinants*

To examine if any of the five immunogenic peptides contains dominant T-cell determinants, CBA/J mice were s.c. primed with 100 μg of intact Tg in CFA as above and 9 days later the draining LNC were cultured in the presence of Tg or free peptide. As shown in (Figure 3.3), Tg-primed LNC responded strongly to Tg in vitro but failed to respond to equimolar (0.2 – 0.9 μM range) concentrations of free peptide. This lack of responsiveness was observed even at higher concentrations of free peptide (up to 75 μM), thus excluding the possibility that a narrow range of peptide concentration might influence the results. Conversely, LNC primed in vivo with any of the five immunogenic peptides did not respond to intact Tg in recall assays in vitro (data not shown). These results do not support the view that these immunogenic peptides encompass dominant T-cell epitopes.

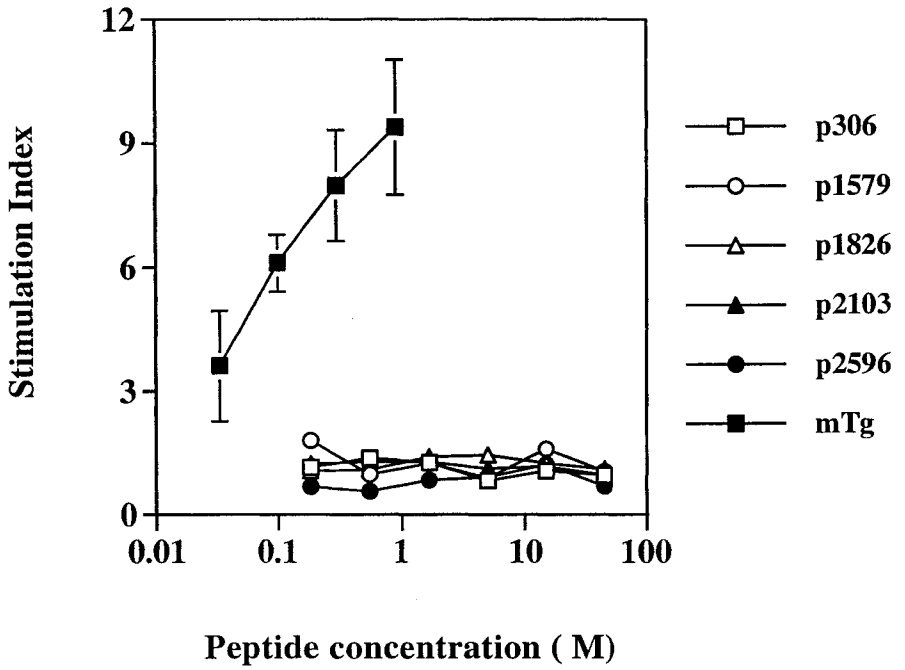


Figure 3.3 Proliferative reponses of mTg-primed LNC to the indicated antigens in vitro.

Mice were injected s.c. with 100μg of Tg in CFA and 9-10 days later draining LN were collected. Cells were adjusted at 4 x 10^6 cells/ml and plated in the presence or absence of titrated amounts of Tg or Tg peptides. Antigen specific proliferation was measured by [^{3}H] uptake as described in Materials and Methods. Data show the mean +/- S.D. of S.I. values of triplicate wells and are representative of 2 experiments. Background cpm was 2000.

3.3.4 *The I-A^k binding peptides are thyroiditogenic in CBA/J mice*

Subsequently, CBA/J mice (6 per group) were immunized with each of the 5 immunogenic peptides above in CFA and were boosted, 3 weeks later, with the same peptide in IFA. Five weeks from the initial challenge, thyroid glands were removed for histological examination of EAT development and the infiltration index was scored as described in Materials and Methods and representatively shown in (Figure 3.4). With the exception of p2102 which did not elicit detectable pathology, all other peptides induced mild and variable EAT with mean I.I. varying from 0.2 (p1826) to 1.2 (p306) (Table 3.2). On the other hand, adoptive transfer of peptide-primed LNCs into syngeneic CBA/J hosts increased both the incidence and the severity (I.I. 0.7 –2.2) of EAT in all groups, including the one challenged with p2102, (Table 3.2), confirming that all five immunogenic peptides have thyroiditogenic potential. Intrathyroidal homing of mononuclear cells was specific since analogous infiltration was not observed in liver or kidney samples of these mice (data not shown). Peptide pathogenicity did not correlate with the presence of peptide-specific IgG in pooled sera of CBA/J mice with EAT since only p2102 and p2596 elicited IgG responses (Figure 3.5) In addition, peptide- reactive IgG did not appear to bind to intact Tg suggesting that p2102 and p2596 are either not expressed on the surface of Tg or that they adopt in free

form a conformation different from the one they assume within the intact Tg molecule.

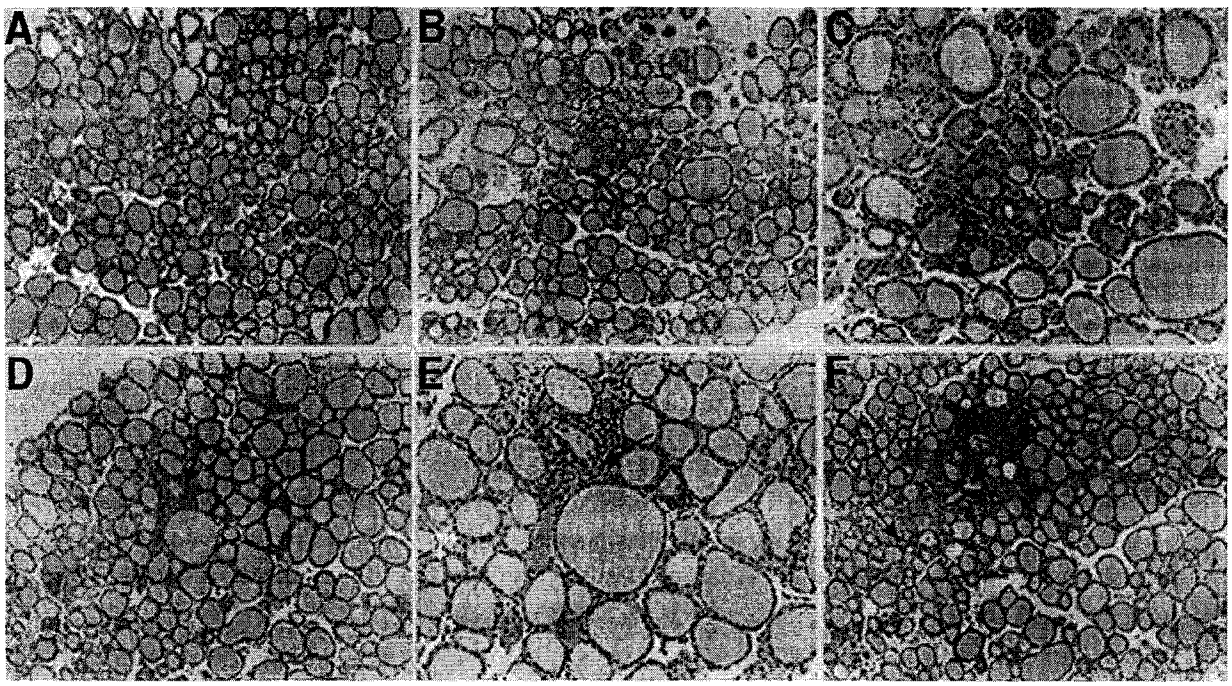


Figure 3.4 Histological appearance of mononuclear cell infiltration in mouse thyroids following induction of EAT with Tg peptides.

A, normal gland I.I.=0; B and C interstitial accumulation of inflammatory cells, I.I.=1; D and E one or two foci of inflammatory cells, I.I. =2 ; F, extensive infiltration, 10-40% of total thyroid gland, I.I.=3. Magnifications : A,B,D and F, 100x; C and E, 200x.

Table 3.2 EAT induction by Tg peptides in CBA/J mice.

	Direct induction of EAT[a]						Adoptive transfer of EAT[b]					
	Infiltration index (I.I.)				# mice	Mean	Infiltration index (I.I.)				# mice	Mean
antigen	0	1	2	3	with EAT	I.I.	0	1	2	3	with EAT	I.I.
p306	3	1	0	2	3/6	1.2 ± 1.5	1	0	1	4	5/6	2.2 ± 1.2
p1579	5	0	1	0	1/6	0.3 ± 0.8	1	0	1	3	4/5	2.2 ± 1.3
p1826	5	1	0	0	1/6	0.2 ± 0.4	1	2	3	0	5/6	1.3 ± 0.8
p2102	6	0	0	0	0/6	0	3	2	1	0	3/6	0.7 ± 0.8
p2596	2	3	1	0	4/6	0.8 ± 0.8	1	1	3	1	5/6	1.7 ± 1.0

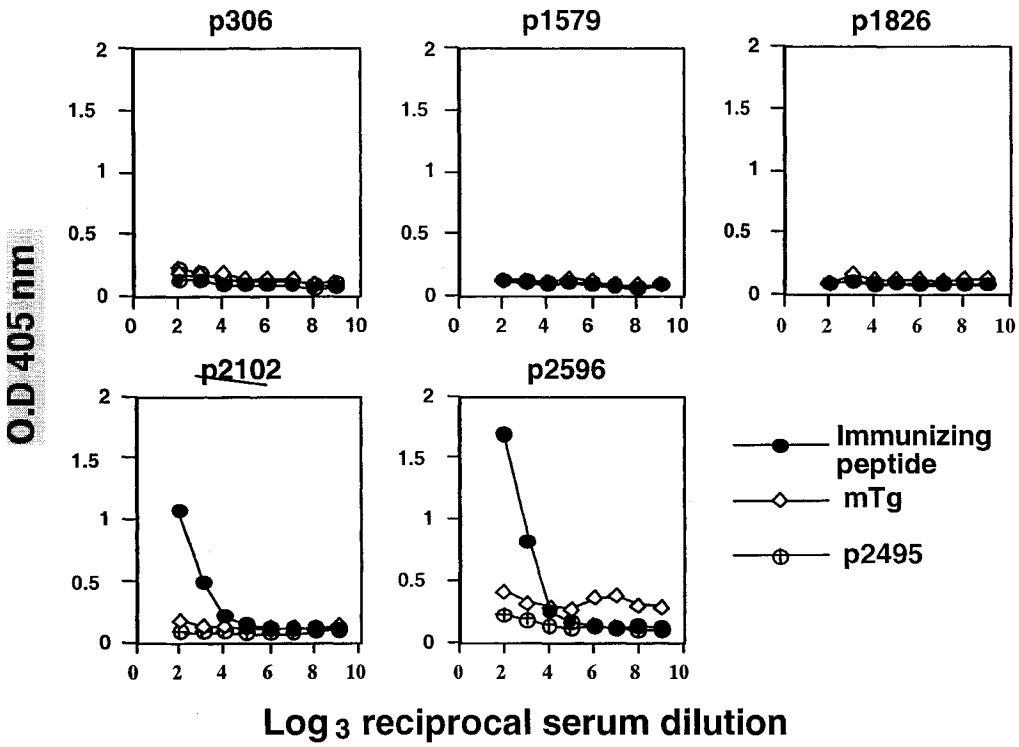


Figure 3.5 IgG responses in pooled immune sera of CBA/J mice.

IgG responses in pooled immune sera of CBA/J mice (6 mice/group) that were primed and boosted with the indicated peptide for EAT development as described in Table II. Mice were bled five weeks after the initial challenge. The reactivities against the immunizing peptide, mTg or p2495 (control) are presented as the mean O.D. values of triplicate wells.

3.4 Discussion

We have conducted an algorithm-based search for pathogenic, I-A^k -binding peptides within the complete mTg sequence in order to substantiate, at the T-cell epitope level, the well known control of Tg-induced mouse EAT by the I-A^k region (Beisel et al. 1982a;Kong et al. 1997). Our findings double the known number of EAT-inducing Tg sequences from five to ten and map, for the first time, four MHC class II-binding pathogenic peptides outside the 534 a.a. C-terminal acetylcholinesterase-homologous region of Tg (Rachinsky et al. 1990). These four peptides (p306, p1579, p1826, and p2102) are scattered throughout the 2200 a.a. N-terminal segment of the Tg molecule (Figure 3.6) which is known to contain three families of homologous domains with cysteine-rich motifs (Malthiery and Lissitzky 1987;Mercken et al. 1985). Interestingly, p306 is localized within the I.4 domain with type 1 homology, whereas p1579 as well as p2102 are encompassed respectively in the IIIa.1 and IIIa.3 domains that express type 3 homology (Table 3.3). The fifth pathogenic peptide p2596 maps in the vicinity of the (2549-2560) epitope which encompasses a thyroxine molecule at position 2553 (Champion et al. 1991).

Table 3.3 Positioning of pathogenic peptides within the repetitive domains of mouse Tg

Domain No.[a]	Mouse Tg Sequence[b]	Amino Acid Coordinates[b]
I.1	F QT VQC QNDGQS CWCVD	29–55
I.2	Y AP VQC DLQRVQ CWCVD	107–123
I.3	F MP VQC 40 CYCAD	170–220
I.4	Y QT VQC QTEGM CWCVD	306–321[c]
I.5	Y ED IQC YAGE CWCVD	606–620
I.6	F LP VQC FNSE CYCVD	673–687
I.7	W RH VQC 124 CWCVD	749–883
I.8	W EP VQC HAGTGQ CWCVD	1018–1034
I.9	Y VR KQT SGTG TWCVD	1095–1109
I.10	F SP VQC DLAQGS CWCVL	1157–1173
IIIa.1	DSPLVQ CL TD CA NDEA	1576–1591[c]
IIIa.2	EQANLW CL SR CA QEPI	1864–1879
IIIa.3	SMAQDF CL QQ CS RHQD	2102–2116[c]

[a]Domain assignment was as described by Malthiery et al. (Malthiery and Lissitzky 1987) for the human Tg sequence. Alignment is shown only for parts of the repetitive type I and type III domains according to (Malthiery and Lissitzky 1987).

[b]Amino acid sequence data and numbering are as described by Kim et al. (Kim et al. 1998).

[c]Peptides used in this study are underlined. Conserved amino acids are shown in bold

The reasons for the lack of immunogenicity of p110, p226, and p2026 are not known but may relate to non H-2 effects on immune responsiveness or influences of motif-flanking residues on T-cell recognition (Vacchio et al. 1989). In competitive inhibition experiments using a cloned A^k-restricted T cell hybridoma specific for the previously identified Tg (2499-2507) epitope (Rao et al. 1994), we have not seen large differences in A^k-binding capacity among all peptides tested (data not shown). Therefore, collectively, the results confirm the predictive value of the algorithm by Altuvia et al (Altuvia et al. 1994) in the identification of A^k-binding peptides. This method relies on physical-chemical and structural properties of peptides such as hydrophobicity, charge, hydrogen bonding capability, etc. that can be extracted from a.a. sequence data. Although in this study we concentrated on candidate peptides with completely overlapping A and B motifs, it is noteworthy that ninety nine additional Tg sites with non overlapping A or B motifs were also revealed by this search (data not shown). In contrast, scanning of the complete mTg sequence with a more restrictive nonamer A^k-binding motif derived from crystallographic data of the A^k molecule complexed with a hen egg lysozyme peptide (Fremont et al. 1998) identified only three sequences: (1576-1584) and (1585-1593) partially overlapping with p1579 and (2107-2115) partially overlapping with p2102 (Table 3.1). Thus, it is difficult to predict from such data how many additional A^k-

binding peptides are harbored within Tg and it is likely that alternative approaches might still identify other EAT-causing determinants. Interestingly, none of the five pathogenic Tg peptides described herein overlap with seven putative I-E^k-binding peptides uniquely expressed in murine Tg, as identified by the EpiMer algorithm (Caturegli et al. 1997). In contrast, p306 and p2102 are being indicated as sites that contain one out of five E^k-binding motifs according to the algorithm of Altuvia et al. (Altuvia et al. 1994).

The current data also bring into focus the fact that none of the seven known A^k-restricted pathogenic Tg peptides - five from the present study, plus the (2499-2507) and (2549-2560) epitopes (Champion et al. 1991;Rao et al. 1994) – can be classified as immunodominant since they cannot be generated following processing of intact mTg in vivo and/or in vitro. This is a rather paradoxical finding in view of the fact that Tg-induced EAT is under control of the I-A^k locus (Beisel et al. 1982a;Kong et al. 1997), implying the presence of dominant A^k-restricted T-cell epitopes in this large autoantigen. The following explanations can be proposed to account for this apparent discrepancy: First, one could maintain that immunodominant T-cell epitopes in Tg exist but remain unidentified because: a) the sheer size of Tg prevents their easy detection, b) such epitopes do not contain hormonogenic sites but are normally iodinated, c)

detection is precluded by limitations of algorithm-based approaches in epitope mapping (here and in (Carayanniotis et al. 1994;Chronopoulou and Carayanniotis 1992) or inherent restrictions in using cloned Tg-reactive T-cell hybridomas for screening the antigenicity of overlapping Tg peptides - a method followed by Champion et al. for the discovery of the pathogenic (2549-2560) sequence (Champion et al. 1991). Second, the theoretical possibility exists that all A^k-restricted Tg peptides interact with MHC with similar affinity and activate T-cell precursors of low frequency, thus not allowing a clearcut hierarchy of immunodominance to emerge. In this case, A^k-controlled susceptibility to EAT or reactivity to mTg in vitro would be detected as a result of additive or synergistic effects of Tg peptide-specific T cell clones which, nevertheless would remain individually undetectable due to their low frequency. Third, and most likely, the conventional criteria for the definition of immunodominance might not apply to Tg. This is an explanation we have previously elaborated on (Carayanniotis and Rao 1997), as it is conceivable that in vitro processing of this large autoantigen does not normally generate enough of any given epitope to activate peptide-specific lymphoid cells. For example, optimal processing of 100 μg/ml (150 nM) of intact dimeric Tg would not generate more than 0.6 μg/ml (300 nM) of any given 2 kDa peptide.

For most Tg peptides tested in this study, the adoptive transfer protocol led to EAT with higher incidence and severity than that induced following direct challenge of hosts with peptide in CFA. These data might be explained on the basis of a peptide dose constraint on direct EAT induction – (100 nmol of peptide may be a relatively low dose given the non dominant nature of these antigens) - vis-a-vis the capacity of preformed peptide-specific effector T cells to home selectively to the thyroid following the adoptive transfer protocol. In analogy with the peptide (2549-2560) previously identified by Roitt's group (Hutchings et al. 1992), the pathogenicity of p2102 was shown only by adoptive transfer of p2102-primed LNC to syngeneic naïve CBA mice, confirming the view (Carayanniotis and Rao 1997) that the thyroiditogenicity of candidate peptides should not be examined only by direct challenge of host mice with the respective epitope. We cannot explain why peptide p2102 does not elicit detectable levels of IFN-γ in culture. However, its potential to elicit mild EAT via the adoptive transfer protocol indicates that p2102 activates a very low number of Th1 cells and /or other types of effector cells that can infiltrate the thyroid. Indeed, previous studies have shown that mice lacking the IFN-γ receptor gene are able to develop disease upon challenge with Tg (Alimi et al. 1998a), and adoptive transfer of Tg-activated T-cells along with a IFN-γ -specific Ab into naïve hosts results in the induction of granulomatous EAT (Stull et al. 1992).

The present findings support the notion that Tg encompasses many non iodinated T-cell epitopes which can be cryptic but pathogenic under conditions that allow their generation and presentation in professional APC. In that regard, we have previously shown that processing of highly iodinated Tg (Dai et al. 2002) or Tg-Ab complexes (Dai et al. 1999) in APC allows selective presentation of pathogenic but cryptic Tg peptides. These mechanisms would promote epitope spreading (Cibotti et al. 1992;Lehmann et al. 1992) and rapid emergence of thyroiditogenic T cells since it is unlikely that peripheral tolerance would have been pre-established against cryptic Tg determinants (Cibotti et al. 1992). Some epitopes may even promote expansion of autoreactive T cells via molecular mimicry with foreign pathogens as has been shown previously with the pathogenic Tg (2695-2706) determinant that exhibits high homology with the (368-381) peptide of murine adenovirus type 1 sequence (Rao et al. 1999). On the other hand, an immunoregulatory role for peptide-reactive IgG Abs is quite unlikely for the Tg sequences described herein, since only p2102 and p2596 elicited IgG responses and these Abs did not bind to intact Tg.

In conclusion, the present study has established the immunopathogenic potential of five novel Tg peptides in CBA/J mice out of a list of eight candidate

sequences that contain A^k-binding motifs. The overall importance of all sequences in mouse EAT can be further evaluated in future studies using H-2^k strains of diverse non H-2 backgrounds and/or strains of other H-2 haplotypes. Work with previously identified pathogenic Tg epitopes, such as the 9-mer (2496-2504) which binds to non-isotypic I-E^k and I-A^s determinants (Rao et al. 1994) and the 11-mer (2549-2559) which induces EAT in CBA/J but not in DBA/1 (H-2^q) or SJL (H-2^s) mice (Rayner et al. 1993) suggests that the outcome of such studies is impossible to predict. However, our findings demonstrate a promising approach for the identification of immunopathogenic Tg peptides with MHC class II-binding properties in EAT which can be analogously applied in the search of Tg epitopes relevant to human thyroiditis.

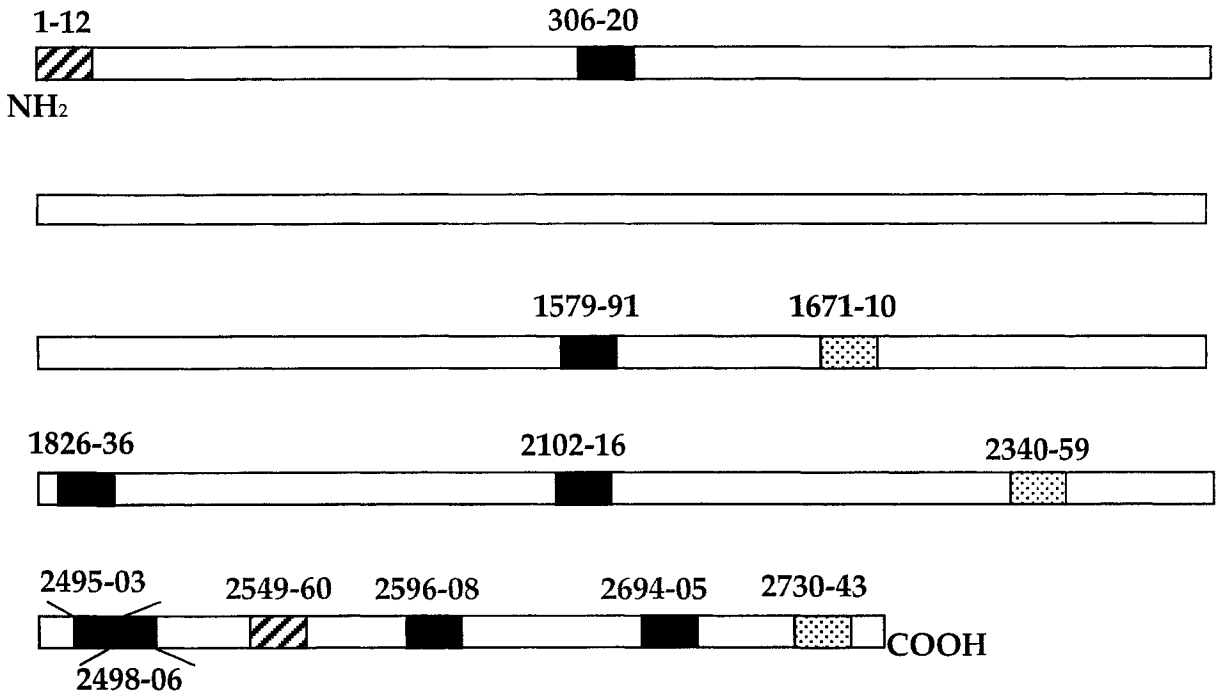


Figure 3.6 Relative positions of Tg peptides that are known to cause EAT.

(▨) Peptides containing hormonogenic sites and shared between mTg and hTg sequence; (■) peptides derived from mTg; (▩) peptides derived from hTg. The a.a. co-ordinates have been assigned according to mTg sequence data by Kim et al. (Kim et al. 1998) and the revised hTg sequence data by van de Graaf et al. (van de Graaf et al. 2001)

4 Chapter 4

Experimental Autoimmune Thyroiditis (EAT) Induced by the Thyroglobulin Peptide (2596-2608): Influence of H-2 and Non H-2 Genes.

4.1 Abstract

We have previously identified five thyroglobulin (Tg) peptides with A^k-binding motifs that induce experimental autoimmune thyroiditis (EAT) in CBA/J ($H\text{-}2^k$) mice. In this study, we have examined whether H-2 or non H-2 genes can influence the immunopathogenicity of peptide p2596 (a.a. 2596-2608), which earlier elicited considerable pathology in CBA/J hosts. The p2596 peptide induced mild EAT- (infiltration index range = 1-2) - in H-2-compatible AKR/J, B10.BR, and C3H/HeJ mice. Moreover, p2596-primed LNC from these mice exhibited peptide-specific proliferative responses and secreted significant amounts of IL-2 and IFN-γ in recall in vitro assays. Priming and boosting of these strains with p2596 resulted in the generation of specific IgG responses five weeks after the initial challenge. In contrast, s.c. challenge of H-2-incompatible strains such as DBA/1J ($H\text{-}2^q$), SJL ($H\text{-}2^s$), DBA/2J ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) with the same peptide dose did not elicit EAT pathology and peptide-specific B- or T-cell responses. These data demonstrate the thyroiditogenic potential of p2596 in $H\text{-}2^k$ strains of diverse non-H-2 backgrounds but not in mice carrying H-2 $^{b, d, q \text{ or } s}$ haplotypes.

4.2 Introduction

Murine experimental autoimmune thyroiditis (EAT), induced with thyroglobulin (Tg) in adjuvant, is under the control of H-2 genes (Vladutiu and Rose 1971) and studies with intra-H-2 recombinant mice have mapped control of disease susceptibility in the H-2A region (Beisel et al. 1982a;Tomazic et al. 1974). Non H-2 genes (e.g. from the C3H background) have also been shown to influence both the incidence and severity of EAT (Beisel et al. 1982b). Overall, the H-2 effects on Tg-induced EAT are demonstrable in a "gradient" pattern: for example, $H\text{-}2^{k,s}$ mice are susceptible, $H\text{-}2^{q}$ mice are moderately susceptible and $H\text{-}2^{b,d}$ mice are resistant to EAT development (Vladutiu and Rose 1971). However, even EAT-resistant strains develop a low degree of thyroiditis following challenge with Tg (Beisel et al. 1982a;Vladutiu and Rose 1971), suggesting that some pathogenic Tg peptides can be presented in the context of "low responder" H-2 genes.

So far, thirteen pathogenic peptides encompassing T-cell epitopes have been identified within Tg, and none of them has been classified as dominant (reviewed in (Carayanniotis 2003)). At least six distinct A^{k}-restricted determinants – five of which have been recently mapped (Verginis et al. 2002)- are contained within these sites. Genetic studies with other Tg peptides,

however, have confirmed that it is impossible to predict which MHC class II alleles are involved in the presentation of a given epitope despite the known genetic pattern of mouse susceptibility to Tg-induced EAT (Carayanniotis and Kong 2000). Thus, the p2694 (a.a. 2694-2711) peptide elicits EAT in H-2^s but not H-2^k mice (Carayanniotis et al. 1994), and vice versa, the p2549 (a.a. 2549 –2560) causes disease in H-2^k but not H-2^s strains (Rayner et al. 1993), although both H-2 haplotypes are susceptible to Tg-induced EAT. No Tg peptides have been yet identified that are pathogenic in "low responder" mice.

In this study, we have examined the pathogenic potential of the recently identified p2596 (a.a. 2596-2608) epitope in H-2- and non H-2-disparate strains. This determinant was chosen because it carries an A^k-binding motif and it causes significant EAT in CBA/J hosts (Verginis et al. 2002). Our goals were: a) to determine whether non H-2 genes could influence the p2596-specific response; and b) whether p2596 could cause EAT in classical "poor responder" strains.

4.3 Results

4.3.1 *Influences of host H-2 and non H-2 genes on the immunogenicity of p2596.*

Mice from H-2^k- haplotype strains (CBA, C3H, B10.BR, AKR), as well as mice from strains carrying other H-2 haplotypes C57BL/6 (H-2^b), DBA/1 (H-2^q), DBA/2 (H-2^d), SJL (H-2^s), were s.c. immunized with 100 nmol p2596 in CFA. Nine days after the challenge, LNC were collected (2 mice per group) and cultured in the presence of titrating amounts of p2596 or equimolar concentrations of another pathogenic Tg epitope (p2494), as a control. Significant p2596-specific proliferation was only observed in cultures with LNCs isolated from H-2^k mice (Figure 4.1). In contrast, LNCs isolated from p2596-primed mice carrying the H-$2^{b, d, q, s}$ haplotypes were unresponsive in the presence of p2596 in vitro (Figure 4.1). LNCs isolated from all eight strains strongly responded to PPD (data not shown). Significant amounts of IL-2 and IFN-γ - but not IL-4 or IL-10 (data not shown)- were detected in culture supernatants of p2596-activated LNCs from H-2^k strains (Figure 4.2) whereas IL-2 or IFN-γ were undetectable in culture supernatants of peptide-primed LNC from non-H-2^k mice. Collectively, these results demonstrate the immunogenicity of p2596 in several H-2^k strains and the lack of its immune recognition in strains carrying other H-2 haplotypes.

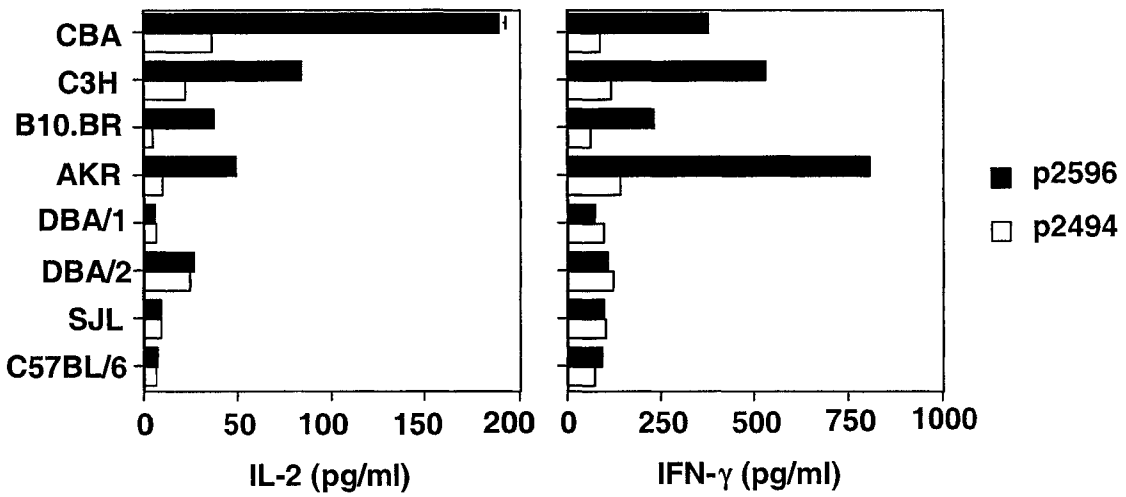


Figure 4.2 Determination of cytokine content in supernatants of p2596-primed LNCs.

Mice were immunized with 100 nmol of p2596 and 9-10 days later draining LNs were collected. Cells were cultured in the presence of 15 μM of the indicated peptide and 48h later culture supernatants were collected. Presence of IL-2 and IFN-γ was determined using sandwich ELISA as described in Materials and Methods. Data represent mean concentration values of duplicate wells extrapolated from standard curves constructed using murine recombinant cytokines. IL-4 and IL-10 were not detected in culture supernatants over a detection limit of 20 pg/ml. Results are representative of three individual experiments. Standard deviations did not exceed 2% of the mean values in all groups.

4.3.2 *The p2596 peptide is thyroiditogenic in H-2^k but not in H-2^{b,d,q,s} mice.*

The profile of p2596 pathogenicity was assessed following s.c priming and boosting of mice from all strains (4-6 mice per group) with the emulsified peptide, as described in Materials and Methods. The thyroid glands were removed 35 days after the initial challenge and examined for signs of mononuclear infiltration. All H-2^k strains developed mild EAT with infiltration indices between 1-2 (Table 4.1). There were no significant differences in the frequency or severity of disease among the different groups. On the other hand, p2596 did not induce any detectable pathology in the thyroids of H-2 $^{b, d, q, s}$ mice in agreement with its lack of immunogenic behaviour in these strains. Analysis of serum samples, obtained at the time of thyroid gland removal, indicated the generation of strong p2596-specific IgG responses in mice bearing the H-2^k haplotype (Table 4.1) but these antibodies were not reactive with intact mTg. In contrast, p2596-specific IgG responses were very low or undetectable in mice of b,d,q, and s haplotypes, in correlation with the LNC response data.

[a]Mice were primed with 100 nmol of p2596 in CFA and boosted three weeks later with 50 nmol of peptide in IFA. Mononuclear infiltration of the thyroid was assessed five weeks after the initial challenge as described in Materials and Methods.

[b]Peptide-specific IgG was assessed in pooled sera by alkaline-phosphatase-based ELISA as outlined in Materials and Methods. The reactivities against p2596, mTg and p2494 are presented as the mean OD values of triplicate wells and were obtained after full titration of sera.

Table 4.1 Immunopathogenicity of p2596 in various strains of mice

Strains	Direct induction of EAT[a]				Serum IgG response (O.D. 405 nm)[b]					
	Infiltration Index (I.I.)				Target antigen					
	0	1	2	# of mice with EAT	p2596		Tg		p2494	
					1:30	1:60	1:30	1:60	1:30	1:60
CBA	1	1	2	3/4	1.13	0.82	0.18	0.19	0.17	0.16
C3H	3	1	1	2/5	0.90	0.36	0.11	0.07	0.09	0.08
B10.BR	4	2	0	2/6	1.27	0.70	0.19	0.16	0.22	0.07
AKR	4	2	0	2/6	1.11	0.48	0.13	0.14	0.17	0.12
DBA/1	6	0	0	0/6	0.20	0.11	0.20	0.16	0.20	0.15
DBA/2	6	0	0	0/6	0.16	0.07	0.15	0.15	0.09	0.16
SJL	6	0	0	0/6	0.33	0.18	0.36	0.16	0.34	0.15
C57BL/6	6	0	0	0/6	0.14	0.20	0.08	0.20	0.16	0.14

4.4 DISCUSSION

Pathogenic Tg peptides have been increasingly used as model antigens in EAT (Carayanniotis 2003;Carayanniotis and Kong 2000) but there is only limited knowledge as to how host genes influence the outcome of the peptide-specific response. Theoretically, for any individual T-cell epitope in Tg, such influences are likely to be more pronounced than those observed against the multi-epitopic intact Tg antigen, leading occasionally to a complete abrogation of the immune response. For example, the peptide (2549-2559) causes EAT in CBA ($H\text{-}2^k$) but not DBA/1 ($H\text{-}2^q$) strains (Rayner et al. 1993) and (2694-2711) is pathogenic in SJL ($H\text{-}2^s$) but not C3H ($H\text{-}2^k$) hosts (Carayanniotis et al. 1994), despite the fact that all four strains are highly susceptible to Tg-induced EAT (Vladutiu and Rose 1971). The peptide p2596 was initially identified as a candidate pathogenic epitope in CBA/J hosts because of the presence of two A^k-binding motifs within the heptamer HGSLELL segment (Verginis et al. 2002). As shown here, p2596 retained its mild immunopathogenicity in three other A^k-expressing strains (C3H, B10.BR and AKR), and this effect was demonstrable at 100 nmol per mouse in accordance with its cryptic nature (Verginis et al. 2002). The p2596-specific proliferative LNC responses and in vitro IL-2 secretion were significantly lower in LNC from B10.BR and AKR vs the CBA mice but this did not correlate with IFN-γ secretion or susceptibility to EAT. Interestingly, C3H non H-2 genes

did not enhance EAT severity and p2596-specific IgG titers, as has been observed for Tg-specific responses in Tg-induced EAT (Beisel et al. 1982a) suggesting that recognition of p2596 does not contribute to this effect in C3H hosts.

Similarly to (2549-2559) (Rayner et al. 1993), p2596 did not elicit EAT in SJL or DBA/1 mice which are very susceptible to Tg-induced EAT. This observation is also in accordance with the non dominant nature of p2596 and the fact that this peptide does not carry MHC-binding motifs that have been described for the I-$A^{b, d, s \text{ and } q}$ and I-$E^{b, d}$ molecules (Brand et al. 1994;Kalbus et al. 2001;Rammensee et al. 1995;Scott et al. 1998;Zhu et al. 2003). However, the immunogenic behaviour of p2596 in these hosts could not have been predicted since the absence of a given motif within a peptide is not necessarily associated with lack of its binding to the respective MHC. For example, as has been mentioned earlier (Verginis et al. 2002), p2596 itself would not have been discovered via an algorithm based on the more restrictive nonamer A^k-binding motif of Fremont et al. (Fremont et al. 1998). In addition, peptides such as the 9mer (2496-2504) are known to bind to non isotypic molecules across H-2 haplotypes (E^k and A^s) (Rao et al. 1994). The lack of immunopathogenicity of p2596 in mice of b,d,q and s H-2 haplotypes may result from lack of MHC binding and/or the absence of available T cell receptors recognizing the appropriate peptide-MHC complex (Dyson et al. 1991;Frankel et

al. 1991). It is noteworthy that none of the three pathogenic Tg peptides that have been tested so far- (2494-2510), (2694-2711) and p2596 - have induced signs of EAT pathology in the "poor responder" BALB/c and B10 strains (Carayanniotis et al. 1994;Chronopoulou and Carayanniotis 1992). The development of mild thyroiditis in EAT-resistant mice after challenge with Tg suggests recognition of certain unknown Tg determinants by these strains. Identification of these determinants would facilitate the study of thyroiditis in genetic backgrounds distinct from those used so far in conventional EAT. As shown here with p2596, the established pathogenicity of certain Tg peptides in genetically diverse hosts would also allow greater flexibility in the design of future EAT studies employing congenic, intra-H-2-recombinant or transgenic mice.

5 CHAPTER 5

Tolerogenic semi-mature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific $CD4^+CD25^+$ T cells

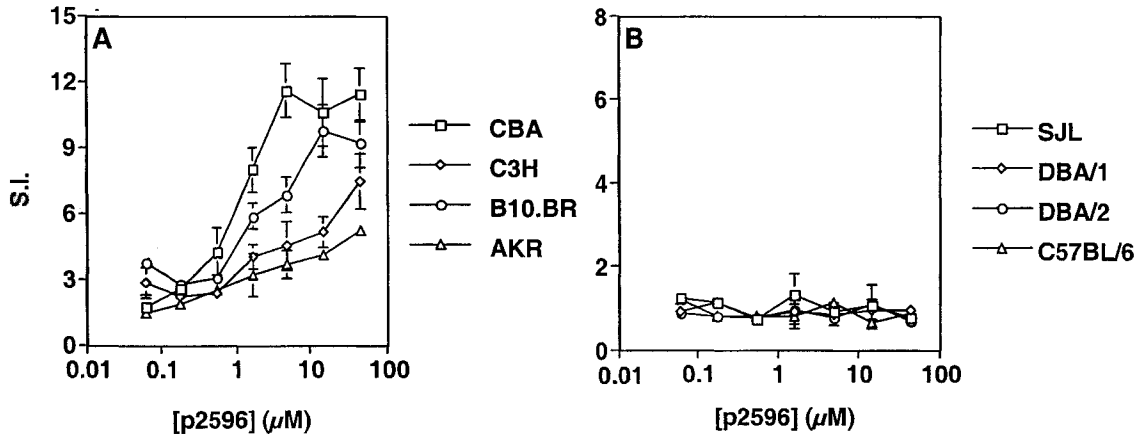


Figure 4.1 Proliferative LNC response to p2596.

H-2^k (A) or non H-2^k (B) mice, were s.c. immunized with 100 nmol of p2596 and 9 days later LNC were collected (2 mice per group) and cultured in the presence of the peptide for 4 days in vitro. [^{3}H]TdR was added during the last 18 h of culture. Background S.I. values were obtained following culture with the control peptide p2494. Results are representative of three individual experiments.

5.1 Abstract

Ex vivo treatment of bone marrow-derived dendritic cells (BM-DCs) with tumor necrosis factor alpha (TNF-α) has been previously shown to induce partial maturation of DCs that are able to suppress autoimmunity. Here, we demonstrate that i.v. administration of TNF-α-treated, semi-mature DCs pulsed with thyrogloblin (Tg), but not with OVA antigen, inhibits the subsequent development of Tg-induced experimental autoimmune thyroiditis (EAT) in CBA/J mice. This protocol activates $CD4^+CD25^+$ T cells in vivo, which secrete IL-10 upon specific recognition of Tg in vitro and express regulatory T cell (Treg)-associated markers such as GITR, CTLA-4 and Foxp3. These $CD4^+CD25^+$ Treg cells suppressed the proliferation and cytokine release of Tg-specific, $CD4^+CD25^-$ effector cells in vitro, in an IL-10-independent, cell contact-dependent manner. Prior adoptive transfer of the same $CD4^+CD25^+$ Treg cells into CBA/J hosts suppressed Tg-induced EAT. These results demonstrate that the tolerogenic potential of Tg-pulsed, semi-mature DCs in EAT is likely to be mediated through the selective activation of Tg-specific $CD4^+CD25^+$ Treg cells and provides new insights for the study of antigen-specific immunoregulation of autoimmune diseases.

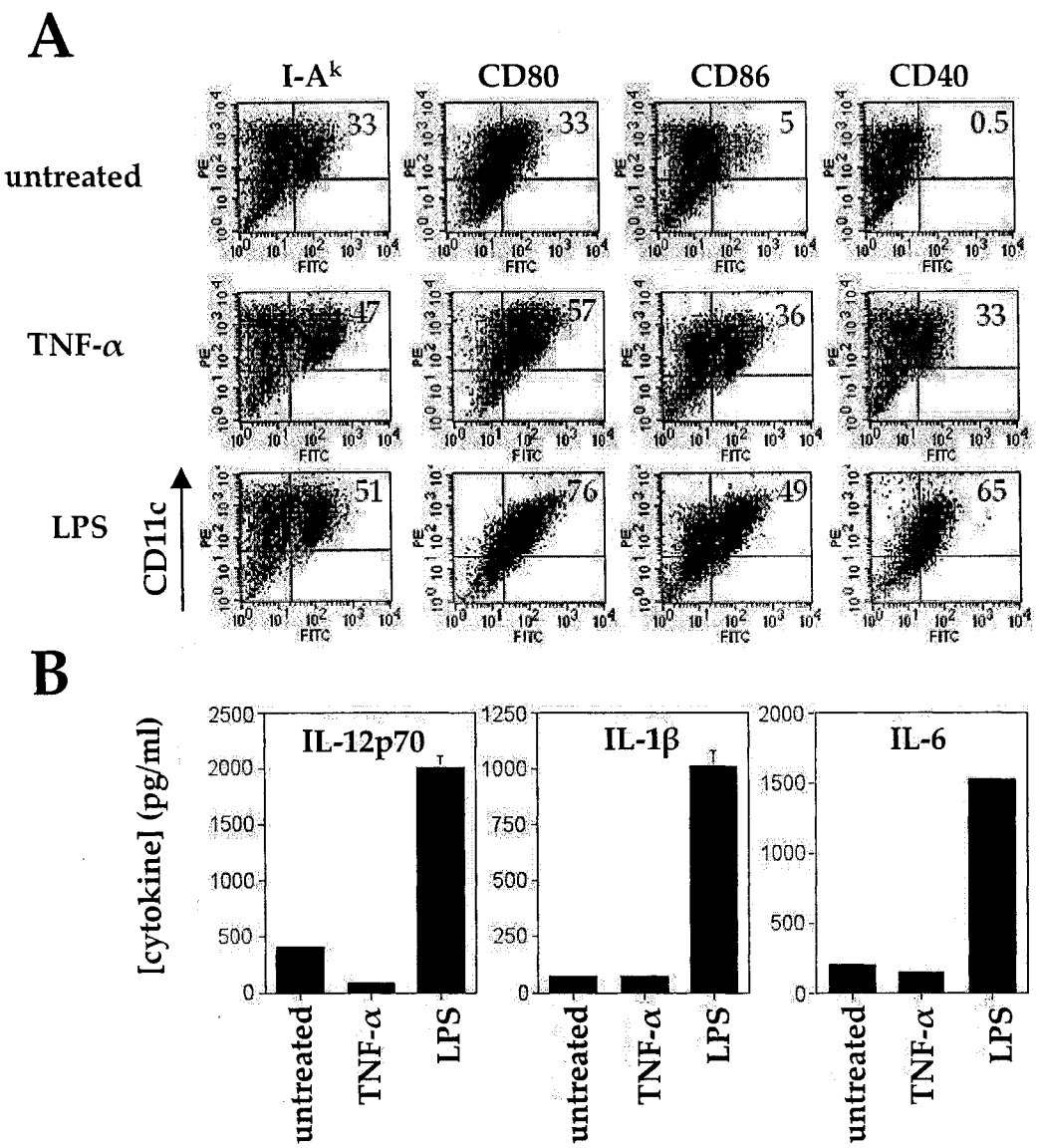
5.2 Introduction

Apart from being highly immunogenic (Banchereau and Steinman 1998), dendritic cells (DC) have been shown to play an important role in peripheral tolerance via various mechanisms, including activation of T regulatory (Treg) cells, induction of T-cell anergy and Th1/Th2 polarization (Jonuleit et al. 2000;Jonuleit et al. 2001;Steinman et al. 2003). Although the exact mechanisms involved in the decision of a DC to become immunogenic or tolerogenic have not been elucidated, increasing evidence suggests that DC function is dependent on their maturation stage (Lutz and Schuler 2002;Mahnke et al. 2002). While immature DCs have been implicated to anergy induction, fully mature DCs are efficient activators of naïve T cells (Steinman and Nussenzweig 2002). An intermediate stage of DC maturation was recently described where DCs express high levels of MHC class II and costimulatory molecules but do not secrete pro-inflammatory cytokines (Menges et al. 2002). This stage of maturation was obtained upon exposure of immature DCs to TNF-α ex vivo, and the cells were termed "semi-mature" DCs. These DCs were shown to induce tolerance through the generation of IL-10-secreting regulatory T (Treg) cells whose profile was not characterized (Menges et al. 2002).

5.3 Results

5.3.1 *Generation of "semi-mature" DCs.*

In order to generate "semi-mature" DCs, we treated BM-DCs with TNF-α for 24h. Untreated and LPS-treated DCs were used as immature and completely mature DCs, respectively. The surface marker phenotype of the DC populations was assessed by flow cytometry. Both TNF-α-treated and LPS-treated DCs expressed higher levels of cell-surface MHC class II, CD80, CD86 and CD40 molecules, as compared to untreated DCs (Figure 5.1). It has been previously shown that TNF-α-treated DCs express low levels of proinflammatory cytokines at the mRNA level and do not secrete significant amounts of IL-12 (Menges et al. 2002). To confirm this finding, we performed sandwich ELISA in DC culture supernatants collected upon 24 h TNF-α treatment. Low levels of IL-12, IL-1β, and IL-6 were secreted by TNF-α-treated DC, similarly to those secreted by immature DCs. In contrast, all cytokines tested were highly produced by LPS-treated DCs (Figure 5.1). Collectively, these results confirmed the generation of "semi-mature" DCs upon TNF-α treatment.


A
I-A^k
CD80
CD86
CD40
untreated
TNF-α
LPS
CD11c
33
33
5
0.5
47
57
36
33
51
76
49
65
PE
FITC
B
[cytokine] (pg/ml)
IL-12p70
IL-1β
IL-6
2500
2000
1500
1000
500
0
1250
1000
750
500
250
2000
1500
1000
500
untreated
TNF-α
LPS

5.3.2 *Induction of Tg-specific, IL-10-secreting $CD4^+CD25^+$ T cells by Tg-pulsed "semi-mature" DCs.*

To assess a possible tolerogenic role of "semi-mature" DCs in EAT, DCs were pulsed with Tg or OVA for 6 h and were subsequently treated with TNF-α for additional 24 h. CBA/J mice received three i.v. injections of DCs (2.5×10^6 cells/mouse) on days 1, 3 and 5 and were sacrificed 21 days after the last challenge. Splenocyte-derived $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were cultured with Tg or OVA and mitomycin C-treated syngeneic splenic APCs as described in Materials and Methods. Both T cell subpopulations did not proliferate in the presence of Ag (data not shown) and they did not produce detectable amounts of IL-2 or IFN-γ in culture supernatants (Table 5.1). However, $CD4^+CD25^+$ T cells, isolated from mice challenged with Tg-pulsed DCs (Tg/DC) secreted significant amounts of IL-10 in response to Tg stimulation in vitro and this effect was Tg-specific since it was not detected in response to OVA. Interestingly, OVA-pulsed DCs (OVA/DC) were not able to induce OVA-specific $CD4^+CD25^+$ T cells with the ability to produce IL-10 (Table 5.1). Additionally, no IL-4 was detected in supernatants of any of the above cell cultures (data not shown), indicating the activation of T cell subpopulation(s) distinct from Th2. These results demonstrate that Tg/DCs have the ability to induce $CD4^+CD25^+$ T cells that produce IL-10 in response to Tg in vitro.

Table 5.1 Induction of Tg-specific, IL-10-secreting CD4+CD25+ T cells following challenge of mice with Tg-pulsed "semi-mature" DCs.

			[Cytokine] (pg/ml)		
In vivo treatment	In vitro activation		IL-2	IFN-γ	IL-10
DC/Tg	$CD4^+CD25^-$	Tg	31 ± 0.3	105 ± 5.3	39 ± 0.4
	$CD4^+CD25^+$	Tg	33 ± 0.1	81 ± 2.1	461 ± 0.4
	$CD4^+CD25^-$	OVA	31 ± 0.1	96 ± 2.1	28 ± 0.7
	$CD4^+CD25^+$	OVA	34 ± 0.2	97 ± 7.2	27 ± 4.3
DC/OVA	$CD4^+CD25^-$	Tg	31 ± 0.2	109 ± 8.4	36 ± 1.8
	$CD4^+CD25^+$	Tg	34 ± 0.2	119 ± 14.6	28 ± 1.7
	$CD4^+CD25^-$	OVA	32 ± 0.3	132 ± 20.6	32 ± 0.2
	$CD4^+CD25^+$	OVA	37 ± 0.4	77 ± 18.2	35 ± 0.8

CBA/J mice were i.v. injected three times (day 1, 3 and 5) with TNF-α-treated DCs pulsed either with Tg or OVA as described in Materials and Methods. Twenty-one days later, spleen-derived $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells (1×10^5 cells/200 μl) were stimulated with Tg or OVA in the presence of mitomycin C-treated syngeneic splenocytes (2×10^6 cells/200 μl) as APC. Culture supernatants were collected at 48 h and assayed for the presence of IL-2, IL-10 and IFN-γ by ELISA. Results are representative of two independent experiments.

5.3.3 *Expression of a Treg phenotype by $CD4^+CD25^+$ T cells*

To examine whether the $CD4^+CD25^+$ T cells obtained from mice challenged with Tg/DC express surface markers associated with Treg, we assessed their phenotype by FACS. The $CD4^+CD25^+$ T cells were $CD62L^{high}$ and $CD69^{low}$ indicating that the cells were not in an activated stage and their expression of GITR, CTLA-4 and CD137 molecules was higher as compared to that of the $CD25^-$ T cell subpopulation (Figure 5.2). Similarly, Foxp3 expression on $CD25^+$ T cells, was 5-fold higher than that in the $CD25^-$ population at the transcriptional level (Figure 5.2). These data indicated that the $CD4^+CD25^+$ T cells isolated from Tg/DC-treated mice possess a phenotype similar to that described for Treg cells (McHugh et al. 2002).

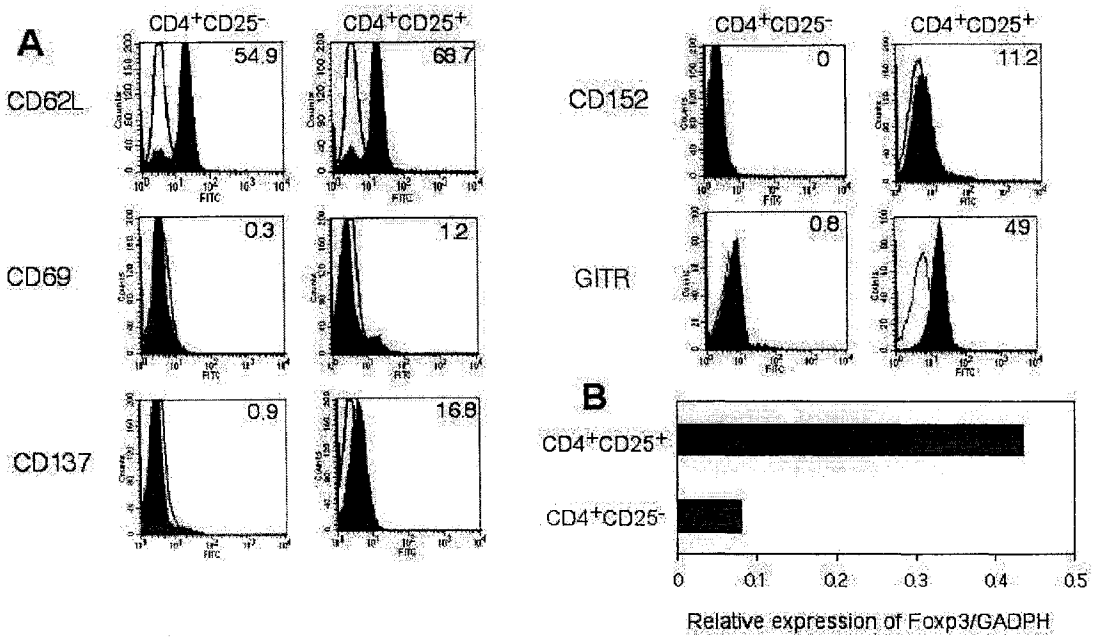


Figure 5.2CD4+CD25+ T cells from Tg/DC-primed mice express the characteristic markers of Treg cells.

$CD4^+CD25^-$ and $CD4^+CD25^+$ splenic T cells were isolated from CBA mice that received Tg-pulsed TNF-α-treated DCs as described in Materials and Methods. A. Cells were stained and analysed for the expression of CD62L, CD69, CD137, CD152 and GITR (filled histograms). Isotype-matched control antibodies are indicated with open histograms. B. RNA was extracted from 5×10^5 cells and reverse-transcribed to cDNA. Foxp3 was amplified by RT-PCR by using gene specific primers. The relative expression of FoxP3 was normalized to the relative expression of GADPH gene.

5.3.4 *$CD4^+CD25^+$ T cells from Tg/DC-challenged mice suppress Tg-specific T cell responses in vitro.*

The functional characteristics of the $CD25^+$ T cell subpopulation were subsequently assessed in mixing experiments. $CD25^-$ T cells isolated from the draining LN of Tg/CFA-immunized CBA mice were used as effector cells. The $CD25^-$ cells proliferated strongly in the presence of Tg and APC and secreted high levels of IL-2 and IFN-γ (Figure 5.3). The response was Tg-specific since proliferation or cytokine release were not detected upon culture of $CD25^-$ cells with OVA (data not shown). $CD4^+CD25^+$ T cells isolated from mice that received Tg/DC, but not OVA/DC, were able to completely suppress the proliferation and cytokine production of the $CD25^-$ effector cells (Figure 5.3). $CD4^+CD25^+$ T cells isolated from naïve mice were also unable to suppress the Tg-specific response in vitro. None of the $CD4^+CD25^+$ T cell populations proliferated in response to Tg and only $CD25^+$ T cells from Tg/DCs-treated mice secreted IL-10 upon culture with Tg and APC, indicating efficient activation of this subset (Figure 5.3). These data highlighted the ability of Tg/DCs to induce a $CD4^+CD25^+$ T cell population that suppress Tg-specific T cell responses.

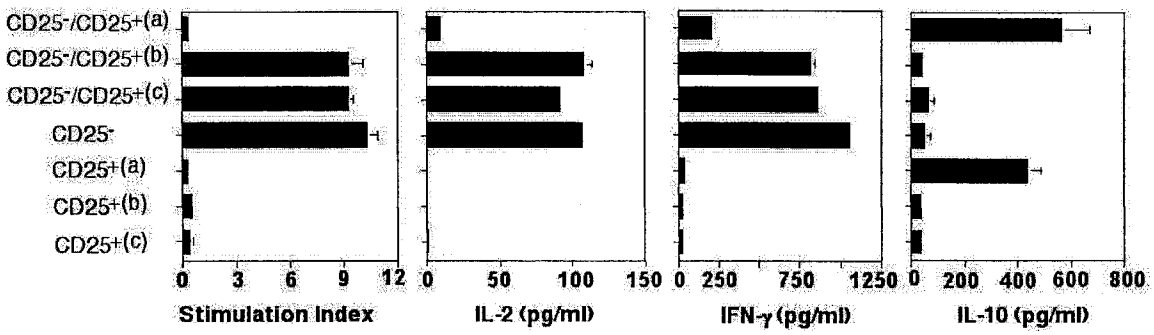


Figure 5.3 Suppression of proliferative Tg-specific T cells by CD4+CD25+ T cells in vitro.

CBA/J mice were immunized s.c. with 100 μg of Tg in CFA and 9 days later $CD4^+CD25^-$ T cells were isolated and used as effector cells. $CD4^+CD25^+$ T cells (Treg) were isolated from spleens from mice that have been injected with [a]Tg-pulsed TNF-α-treated DCs, [b]OVA-pulsed TNF-α-treated DCs, as described in Figure 3 or [c]either isolated from naïve mice. Effector cells (1×10^5 cells/well) were culture with equal numbers of Treg cell in the presence of Tg. Mitomycin-C treated syngeneic splenic cells (2×10^5 cells/well) were used as APC. Data represent stimulation index (S.I.= cpm in the presence of Ag/cpm in the absence of Ag) values of triplicate wells. Secretion of IL-2, IFN-γ and IL-10 was measured by cytokine ELISA in supernatants collected after 48 h of culture. Data are representative of two independent experiments.

5.3.5 *$CD4^+CD25^+$ T cells mediate suppression in a cell-cell contact-dependent manner.*

To investigate the mechanism by which $CD25^+$ T cell mediate suppression, Tg-specific $CD4^+CD25^-$ effector cells were stimulated with Tg and APC in a 24-well transwell plate. Tg-specific $CD25^+$ T cells, isolated as above, were placed in the same well or in the upper chamber at a 1:1 ratio, in the presence (or absence) of Tg. Inhibition of proliferation was observed only when effector cells and $CD25^+$ cells were co-cultured in the same well (Figure 5.4), and this inhibition correlated well with the suppression of IL-2 and IFN-γ secretion by the effector cells. As expected, significant amounts of IL-10 were detected only in wells where $CD25^+$ T cells were cultured in the presence of Tg and APC. These data also suggested that while contact between effector cells and $CD25^+$ cells is required to mediate suppression, it is not needed for the production of IL-10 by Tregs. Altogether, the results supported the view that $CD25^+$ T cells suppress the activation of Tg-specific $CD25^-$ effector cells via a cell-cell contact-dependent, cytokine-independent mechanism.

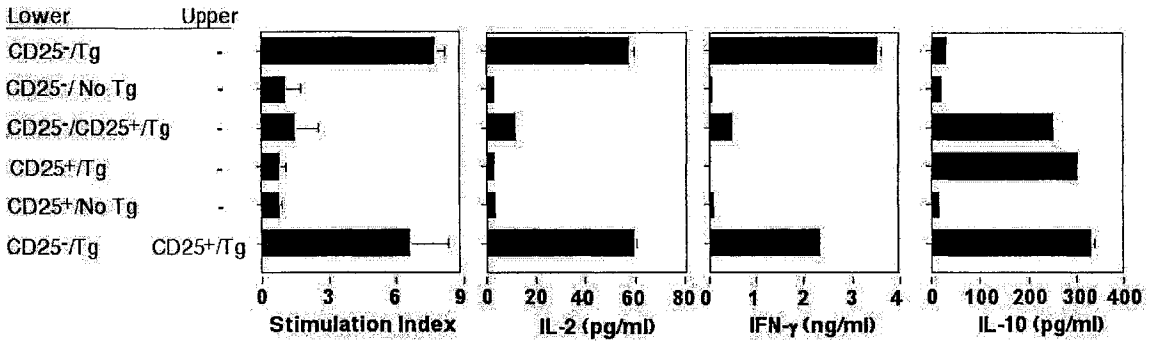


Figure 5.4 Cell-cell contact is required for the suppressive activity of the Tg-specific Treg cells.

Effector and CD4+CD25+ T cells were isolated as in Figure 3. 6 x 10^5 effector cells were stimulated in 24-well transwell plates with 100 μg of Tg and 1.2 x 10^6 APC prepared as described in Figure 4. In addition, 6 x 10^5 Treg were either added directly to the culture or placed in the transwell chamber with Tg and APC. Proliferation was assessed after 4 days of culture. Supernatants were collected after 48 h of stimulation and cytokine ELISA was performed to measure the content of IL-2, IFN-γ and IL-10 in the culture. Data are representative of two independent experiments.

5.3.6 *Tg/DCs can suppress EAT induction through the generation of $CD4^+CD25^+$ T cells.*

To assess the tolerogenic potential of Tg/DC in Tg-induced EAT we challenged CBA mice on days 1, 3, and 5 with Tg/DC, OVA/DCs or PBS and two days after the last DC challenge, we immunized them with Tg in CFA. Twenty-one days later, the thyroids were removed and examined for mononuclear cell infiltration. As expected, mice that received PBS and were subsequently challenged with Tg showed the highest degree of EAT (I.I. = 2.2), (Figure 5.5) whereas mice that received OVA/DCs had a slight decrease of EAT incidence (I.I. = 1.8) that was not statistically significant ($p = 0.613$). In contrast, disease was significantly suppressed (I.I.= 0.5, $p = 0.006$) in mice that received Tg/DCs prior to Tg challenge. We did not detect any thyroid pathology in mice challenged with Tg/DC or OVA/DCs alone, indicating that the semi-mature DCs themselves did not contribute to the development of EAT. Based on these observations, we formulated the hypothesis that Tg/DCs but not OVA/DCs are able to expand $CD4^+CD25^+$ T cells with the ability to suppress Tg-induced EAT. To directly address this, we adoptively transferred $CD25^+$ T cells into naïve mice and one day later, we challenged the mice with Tg in adjuvant. Mononuclear cell infiltration of the thyroid was assessed twenty-one days later, as described in Materials and Methods. Mice that received $CD25^+$ cells from Tg/DCs-treated

donors showed a significant decrease of EAT (I.I. = 0.89, p =0.03) (Table 5.2), as compared to the control group (I.I. = 2.5). On the other hand, EAT in mice that received $CD25^+$ T cells from OVA/DC-challenged mice was not significantly reduced (I.I. =2.13, p=0.50), as compared to that of control mice immunized with Tg/CFA alone. Collectively, these data demonstrated the suppressogenic potential of $CD4^+CD25^+$ T cells derived from mice that have been challenged with Tg/DCs, but not OVA/DCs.

$CD4^+CD25^+$ Treg cells arise normally in naïve mice and constitute approximately 10 % of the peripheral $CD4^+$ T cells. Treg cells are generated in the thymus upon high-avidity interactions with self-peptides (Bensinger et al. 2001;Jordan et al. 2001;Seddon and Mason 1999) and they participate in the maintenance of peripheral self-tolerance (Sakaguchi et al. 2001;Shevach 2000). Their development has been shown to programmed by the transcription factor Foxp3 (Fontenot et al. 2003;Hori et al. 2003;Khattri et al. 2003) and, in addition to CD25, they constitutively express CTLA-4 (Takahashi et al. 2000) and GITR (McHugh et al. 2002;Shimizu et al. 2002) on their surface. Although accumulating evidence suggests a major role of this subset in the maintenance of self-tolerance (Sakaguchi et al. 2001), the antigen specificity and the exact mechanism(s) of action of the $CD4^+CD25^+$ T cells remain unresolved. Meanwhile, ample evidence indicates that DCs play an important role in expansion/induction of Treg cells (Jonuleit et al. 2001) that could be specific for self antigens (Fisson et al. 2003). However, a clear profile of DC involved in this process has yet to be identified.

EAT, a murine model of Hashimoto's thyroiditis in humans, can be induced upon challenge of susceptible animals with thyroglobulin (Tg) in CFA (Charreire 1989). The disease is mediated by $CD4^+$ T cells and characterized by lymphocytic infiltration of the thyroid gland (Weetman and McGregor 1994). Several reports

have suggested a major role of the $CD4^+CD25^+$ T cells in immunoregulation of EAT. First, elimination of Treg cells in mice resulted in development of multi-organ autoimmune diseases including thyroiditis whereas reconstitution of $CD4^+CD25^+$ T cells inhibited development of autoimmunity (Sakaguchi et al. 1995). Second, it was recently reported that $CD4^+CD25^+$ T cells, isolated from GM-CSF-treated mice, were able to suppress Tg-specific T cell responses in vitro (Vasu et al. 2003). The authors suggested differential activation of DCs by GM-CSF that induces Treg cells. In addition, Kong and coworkers showed that $CD4^+CD25^+$ T cells from Tg-tolerized mice can suppress mouse Tg-specific responses in vitro (Morris et al. 2003).

In this study, we sought direct evidence for the induction of $CD4^+CD25^+$ Treg cells, with the ability to suppress Tg-induced EAT. To investigate this, TNF-α-treated DCs isolated from CBA/J mice were pulsed with Tg and transferred into syngeneic mice. Splenocyte-derived $CD4^+CD25^+$ T cells were tested for their ability to suppress Tg-specific effector T cell responses in vitro as well as development of EAT.

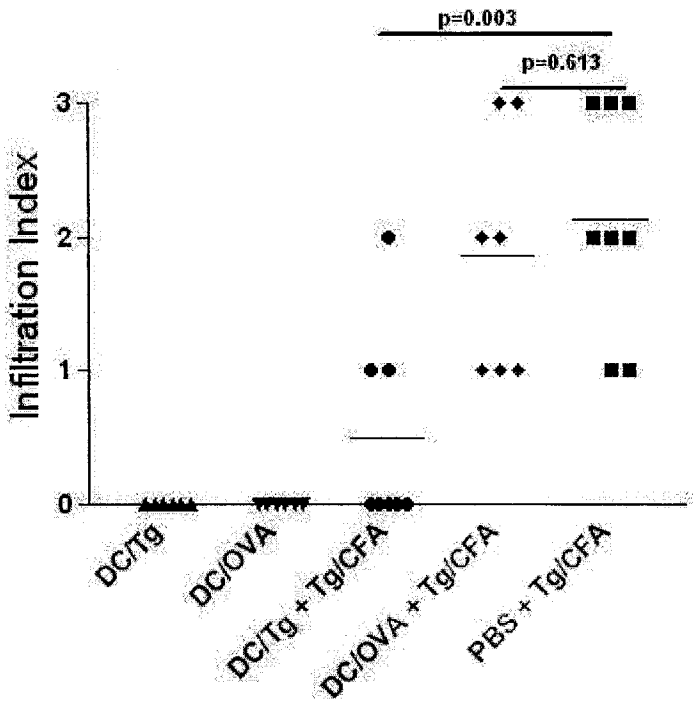


Figure 5.5 Suppression of Tg-induced EAT by Tg-pulsed smDCs.

CBA/J mice received three i.v. injections (day 1, 3 and 5) of 2.5 x 10^6 TNF-α-treated DCs pulsed with 100 μg of Tg or OVA. Control mice received three i.v. injections of PBS. Two days after the last DC injection some mice were immunized with 100 μg of Tg in CFA. On day 21, all mice were sacrificed and thyroid glands were collected. Mononuclear cell infiltration index (I.I.) was scored as described in Materials and Methods. Statistical analysis was performed using the Mann-Whitney nonparametric test.

Table 5.2 Adoptive transfer of CD4+CD25+ T cells from Tg/DC-treated CBA mice into naïve hosts inhibits EAT development.

		Infiltration index (I.I.)						
DC challenge	Antigen in vivo	0	1	2	3	# of mice with EAT	Mean of I.I.	p value
Tg/DC	Tg	5	2	1	0	3/8	0.50	0.006
OVA/DC	Tg	0	3	2	2	7/7	1.86	0.613
None	Tg	0	2	3	3	8/8	2.13	
Tg/DC	None	6	0	0	0	0/6	0	
OVA/DC	None	6	0	0	0	0/6	0	

5×10^5 CD4+CD25+ T cells isolated from either [a]Tg-pulsed DCs– or [b]OVA-pulsed DCs –primed CBA mice were adoptively transferred i.p. into synjeneic naïve recipients. Control mice received one i.p. injection of PBS. One day after the T cell transfer some mice were immunized with 100 μg of Tg in CFA. On day 21 all mice were sacrificed and thyroid glands were collected. Mononuclear cell infiltration index (I.I.) was scored as described in Materials and Methods. Statistical analysis was performed using the Mann-Whitney nonparametric test.

5.4 Discussion

The present study demonstrates that Tg-pulsed "semi-mature" DCs can induce Tg-specific $CD4^+CD25^+$ T cells with the ability to inhibit EAT development. Our data confirm earlier findings in the EAE model that TNF-α-treated DCs, expressing a "semi-mature" phenotype, mediate antigen-specific protection against autoimmune disease (Menges et al. 2002), and extend these observations by highlighting that this protection is likely to be mediated by autoantigen-specific $CD4^+CD25^+$ Treg cells. Tg/DC, but not OVA/DC, may activate and/or expand pre-existing, naturally occurring, Tg-specific $CD25^+$ T cells that have been positively selected in the thymus. This hypothesis is in agreement with recent data demonstrating that thymic expression of a self-antigen facilitates the development of high numbers of antigen-specific Treg cells (Jordan et al. 2001;Walker et al. 2003) and that Tg is known to be expressed intrathymically (Heath et al. 1998).

Although the critical factors that determine the tolerogenic potential of TNF-α-treated DCs cells remain unknown, the absence of proinflammatory cytokine secretion by this subset has been suggested to contribute to tolerogenicity (Lutz and Schuler 2002). This hypothesis is supported by our

findings since the TNF-α-treated DCs were found to secrete low levels of IL-12, IL-6 or IL-1β. Unlike the immature DC, semi-mature DCs also express relatively high levels of costimulatory molecules such as CD80, CD86 and CD40 which have been implicated to play an important role in homeostasis and expansion of $CD4^+CD25^+$ Treg cells. Blockage of CD80/86 molecules has been known to lead to autoimmunity (Lohr et al. 2003;Salomon et al. 2000) and CD40-deficient mice exhibit a reduced population of $CD25^+$ T cells associated with increased T cell autoreactivity (Kumanogoh et al. 2001). In contrast to the above findings, two groups have recently reported that mature DCs are able to expand $CD4^+CD25^+$ Treg cells both in vitro and in vivo (Oldenhove et al. 2003;Yamazaki et al. 2003). However, the classification of DCs as mature in both studies was based on their surface phenotype expression and not on the cytokines that they secrete.

Apart from TNF-α, several other factors have been described to induce DCs with a semi-mature-like phenotype, including lactobacilli (Christensen et al. 2002), and cholera toxin (Braun et al. 1999). It has also been proposed that steady-state migrating DCs in vivo ("veiled" cells) resemble the ex-vivo generated semi-mature DCs (Lutz and Schuler 2002). Veiled cells circulate through peripheral tissues where they pick up apoptotic cells and migrate to the secondary lymphoid organs (Huang et al. 2000;Inaba et al. 1998), where they present self-

peptides in the context of MHC molecules (Scheinecker et al. 2002). In the absence of maturation stimuli, however, DCs do not secrete proinflammatory cytokines and induce tolerance (Hawiger et al. 2001;Liu et al. 2002;Steinman and Nussenzweig 2002). Other types of DCs have been shown to induce tolerance by distinct mechanisms (Jonuleit et al. 2001). For example, human IL-10-treated DCs were characterized by low levels of MHC class II and costimulatory molecule expression and induced antigen-specific anergic T cells (Steinbrink et al. 1997;Steinbrink et al. 1999). In addition, mouse pulmonary DCs that secrete IL-10, but not IL-12, upon exposure to antigen, induced tolerance through the development of Treg cells (Akbari et al. 2001). Tolerogenic DCs have been generated by other methods including treatment with vitamin D3 (Penna and Adorini 2000), corticosteroids (Rea et al. 2000), and low doses of GM-CSF (Lutz et al. 2000) but it was not clear in these studies how modulated DCs mediated tolerance.

The functional and phenotypic characteristics of the Tg-specific $CD4^+CD25^+$ T cells are consistent with those described for the naturally arising Treg cells (Banz et al. 2003;McHugh and Shevach 2002;Read et al. 2000;Sakaguchi 2004;Shimizu et al. 2002;Takahashi et al. 2000;Thornton and Shevach 1998). Yet, Tg-specific $CD25^+$ Treg cells – maintained presumably by the normal

physiological levels of Tg in the periphery – could not be functionally detected in the spleen of naive CBA/J mice. This suggests that challenge with Tg/DCs must increase the relative number as well as the efficiency of Tg-specific $CD4^+CD25^+$ T cells in a selective manner, since we did not observe a size difference or phenotypic changes in the overall splenic $CD4^+CD25^+$ T cell subset between the Tg/DC- challenged mice and the controls (data not shown). A similar expansion and/or activation of Tg-specific, $CD25^+$ Treg cells, has been proposed to occur in the induction of tolerance observed following the elevation of Tg levels in the circulation (Morris et al. 2003).

Our results do not support a role for IL-10 in the suppressive effect of Tg-specific $CD25^+$ cells in vitro but cannot formally exclude a possible role for this and other cytokines in the observed suppression of EAT. Vasu et al.(Vasu et al. 2003) have recently shown that GM-CSF-treated mice have increased numbers of $CD4^+CD25^+$ T cells and fail to develop EAT upon challenge with Tg. $CD4^+$ T cells isolated from GM-CSF-treated mice did not proliferate in response to Tg in vitro, but addition of neutralizing anti-IL-10 mAb increased the Tg-specific T cell response, indicating a possible role for IL-10 and an alternative mode of EAT suppression in this model. Currently, the reasons for these contrasting findings remain unclear; previous studies have provided evidence for (Annacker et al.

2001) or against (Suri-Payer et al. 1998;Thornton and Shevach 1998) an involvement of IL-10 in $CD4^+$ $CD25^+$ T cell-mediated suppression.

The observation that $CD4^+CD25^+$ T cells from Tg/DC-treated mice mediate in vitro suppression by a cell contact-dependent mechanism, and with no apparent requirements for secretion of soluble factors agrees well with previous findings (Bluestone and Abbas 2003;Hawiger et al. 2001;Sakaguchi 2004;Shevach 2000;Thornton and Shevach 1998). The CTLA-4 molecule, expressed on the surface of Tg-specific $CD4^+CD25^+$ T cells, has been shown to contribute to the cell-cell contact mediated suppression of effector cells (Takahashi et al. 2000). $CD4^+CD25^+$ Treg cells are also known to mediate suppression through ligation of the cell surface bound TGF-β to TGF-βR on target cells (Green et al. 2003;Nakamura et al. 2001) and additional costimulatory molecules, such as OX40, ICOS and 4-1BB, have been reported to be involved in downregulation of immune responses (Croft 2003). Lastly, in our study, the activation and/or expansion of Treg cells by Tg/DC, but not OVA/DC, suggests that this phenomenon is Tg-specific but it remains to be established whether suppressor effector function is completely antigen non-specific, as has been well described in other systems (Shevach 2000).

The generation of Tg-specific $CD4^+CD25^+$ Treg cells by semi-mature Tg/DCs raises new questions about their fine specificity and mode of function. Currently, thirteen pathogenic epitopes have been mapped within the Tg molecule (Carayanniotis 2003) and it will be interesting to test whether effector and Treg cells recognize distinct or overlapping regions in mouse Tg as well as examine if post-translational modifications of Tg have any role in this process. The delineation of physiological processes that promote the generation of tolerogenic DC in EAT, similar to TNF-α-treated DCs, would contribute greatly to our understanding of the immunoregulation of this disease. Lastly, the examination of the effectiveness of $CD25^+$ Treg cells in reversing established disease would aid in the development of new therapeutic approaches in this field.

Generation of T cell receptor (TCR)-transgenic mice to study experimental autoimmune thyroiditis (EAT).

6.1 Abstract

EAT can be induced after challenge of SJL mice with the thyroglobulin peptide p2496 in adjuvant. We are in the process of generating TCR transgenic mice with TCR-α and -β chains derived from the p2496-specific I-A^s-restricted, 9.13 T-cell hybridoma clone. By RT-PCR and subsequent cloning and sequencing of the amplified product it was revealed that the 9.13 T cell hybridoma expresses a rearranged Vβ4-Dβ2.1-Jβ2.1 chain. The 9.13 TCR α chain was identified by "anchor PCR". The amplified product was cloned and sequenced to reveal that the 9.13 T cell hybridoma utilizes the rearranged Vα1.1-Jα49 chain. Genomic DNA isolated from the 9.13 clone, was PCR-amplified with appropriate primers to introduce unique restriction sites for subsequent cloning into pTα and pTβ expression vectors that have been engineered to contain TCR regulatory elements, as well as the Cα and Cβ regions. Following digestion with specific restriction enzymes to remove prokaryotic sequences, linearized fragments were injected into fertilized C57/BL6 x SJL oocytes. Integration of the transgene was screened by PCR amplification of genomic DNA with VJα and VDJβ specific primers. Positive mice were backcrossed to SJL mice for at least five generations. These mice will be valuable tools to study parameters (such as iodination of Tg or high iodine intake) that promote the generation of this epitope *in vivo* and lead to EAT development.

6.2 Introduction

It has been demonstrated that self-reactive T cells are present in the periphery of healthy individuals (Markovic-Plese et al. 1995) as well as normal animals (Anderson et al. 2000), and their activation is limited by mechanisms of peripheral tolerance induction (Kamradt and Mitchison 2001). Breakdown of one of these mechanisms can lead to activation of self-reactive T cells and development of autoimmune diseases (Kamradt and Mitchison 2001).

Studies on the activation and effector function of self reactive T cells as well as on mechanisms that influence tolerance induction were hampered in the past by the low frequency of T lympocytes specific for a self antigen (one in 10^4 to 10^5) that are present in normal mice (Anderton et al. 1999). This obstacle was circumvented with the generation of transgenic mice that express TCR specific for a given self antigen. The majority of the T cells in TCR transgenic mice are naïve and express TCR with unique specificity for antigen. TCR transgenic mice have been described for most models of autoimmunity including experimental autoimmune encephalomyelitis (EAE) (Goverman et al. 1993;Lafaille et al. 1994), diabetes (Katz et al. 1993;Tarbell et al. 2002), rheumatoid arthritis (Mori et al. 1992;Osman et al. 1998) and autoimmune gastritis (Alderuccio et al. 2000;McHugh et al. 2001), and contributed further in our understanding of the autoimmune processes.

In the field of thyroiditis, transgenic mice expressing TCR specific for a Tg peptide or other thyroid antigens have not yet been described. In this study, we attempted to generate TCR transgenic mice specific for the p2496 Tg peptide that has been shown to be pathogenic in SJL mice (Rao et al. 1994). The α and β TCR chain genes were derived from the p2496-specific, I-A^s-restricted, 9.13 T-cell hybridoma clone and inserted into the pTα and pTβ cassette expression vectors (Kouskoff et al. 1995). These vectors contain the natural TCR α- β-promoter/enchancer elements allowing specific expression of the receptor on the T cell surface (Kouskoff et al. 1995). The 9.13 TCR-transgenic mice will be invaluable tools to study parameters that influence the activation and effector function of p2496-specific T cells.

6.3 Results

6.3.1 *The p2496 peptide specifically activates the 9.13 T-cell hybridoma clone.*

The 9.13 T-cell hybridoma was generated by fusion of LNC obtained from p2496-primed SJL mice (H-2^s), with the BW5147$\alpha^-\beta^-$ (TcR $\alpha^-\beta^-$) myeloma cell line as previously described (Rao et al. 1997). To examine the specificity of the clone, T cells were cultured with an H-$2A^s$-expressing APC cell line (LS102.9) in the presence of p2496 or another Tg pathogenic epitope p2695 (control antigen) as described in Materials and Methods. After 24 h, culture supernatant was collected for assessment of IL-2 release as measured by the proliferation of the CTLL-2 line using [^{3}H] thymidine. Only p2496 and not the control peptide was able to activate the 9.13 T cell hybridoma as indicated from the proliferation of the CTLL-2 cell line (Figure 6.1).

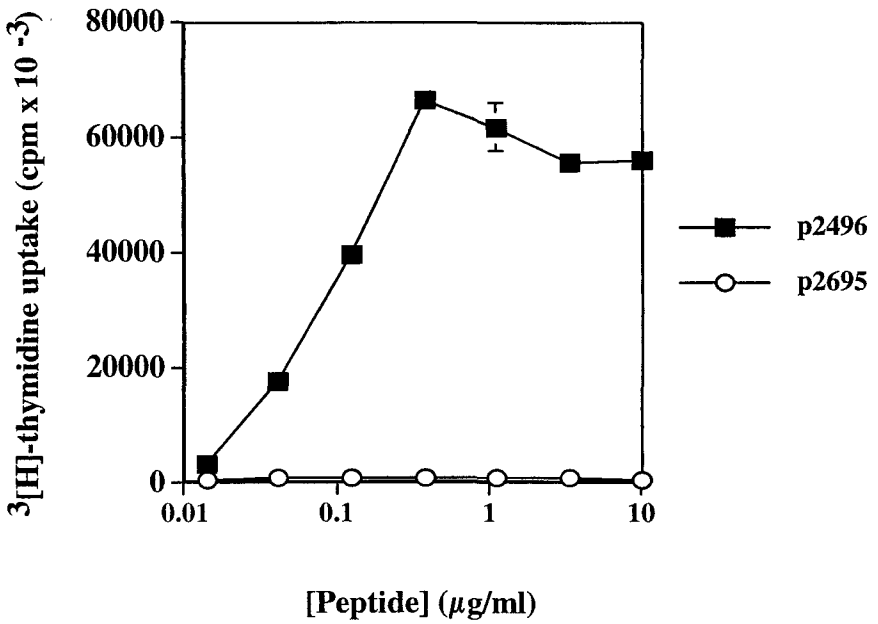


Figure 6.1 Activation of the A^s-restricted 9.13 T-cell hybridoma by the p2496 Tg epitope.

9.13 hybridoma cells (10^5/well) were cultured with equal numbers of the LS102.9 antigen presenting cell line in the presence of either p2496 or p2695 (control) peptides as described in Materials and Methods. The IL-2 content in culture supernatants, collected after 24 hours of culture, was assessed by the proliferation of the IL-2-dependent CTLL-2 cell line.

6.3.2 *Characterization of the α TCR chain gene expressed on the 9.13 T-cell hybridoma.*

Total RNA was extracted from the 9.13 T cell clone and the RML-RACE technique for the identification of the 5′ end of the α TCR chain was performed as described in Materials and Methods. Since the α chain sequence was unknown we could not estimate the exact size of the expected PCR product. By taking into consideration the average length of the published α chain sequences we estimated the approximate length of the amplified α chain gene to be between 600-750 bp or 475-625 bp using the Cα1 or Cα2 reverse primers, respectively. Upon PCR amplification of cDNA using Generacer-F and Cα1-R primers, a strong band close to 700 bp (lane 2) was detected whereas with the same forward primer and the Cα2-R primer a band close to 580 bp (lane 3) was found (Figure 6.2). Based on the size difference of the two PCR products we concluded that these bands should contain the α chain gene of the 9.13 T cell hybridoma. The band amplified with the Cα1 primer (lane 2) was gel purified and inserted into the pCR4-TOPO vector. Plasmid DNA was purified from colonies obtained after transformation of competent cells and was sequenced as described in Materials and Methods. Analysis of the sequence results revealed that the TCR utilizes an α chain gene of the Vα1.1 family rearranged with Jα49 joining sequence (Figure 6.3).

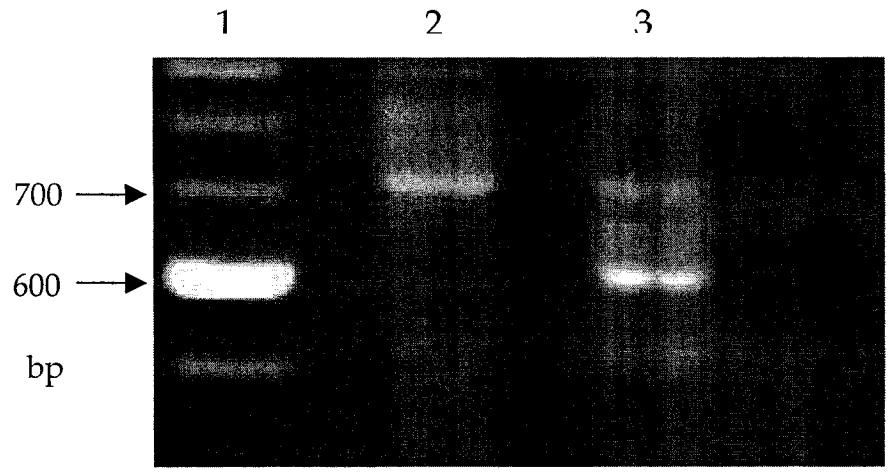


Figure 6.2 Amplification of the TCR-α chain gene from the 9.13 T cell hybridoma clone by RT-PCR.

RNA was extracted from the 9.13 T cell hybridoma and cDNA was prepared using the 5′ RACE kit as described in Materials and Methods. RT-PCR was performed using reverse primer specific for the α constant region (Cα1 lane 2 or Cα2 lane 3) and an oligo specific forward primer. PCR products were visualized in 1.5% agarose gel. The 100 bp DNA ladder is shown in lane 1.

ATG AAA TCC TTG AGT GTT TCA CTA GTG GTC CTG TGG CTC CAG GTA

AAC TGT AAG TTT GGG AAT TCC TTT GGG ATC CAG GTG TGA TAT GTA

AAG TTA TTG TCT GCT CCA GGC TAA TGG TAC AAA CAC AGA TGT TCT

TAA TCC CTA GTT AGG GGC GGG AGA CCC ATG GAA ATG CCA TTC TGG

AAT GTT AGC ATC CAT ACG CAT CCA GTC TTG ATT GTC TGA CCT TTG

TTT TTC TGT ACA GGC GTG AGG AGC CAG CAG AAG GTG CAG CAG AGC

CCA GAA TCC CTC AGT GTC CCA GAG GGA GGC ATG GCC TCT TTC AAC

TGC ACT TCA AGT GAT CGT AAT TTT CAG TAC TTC TGG TGG TAC AGA

CAG CAT TCT GGA GAA GGC CCC AAG GCA CTG ATG TCA ATC TTC TCT

GAT GGT GAC AAG AAA GAA GGC AGA TTC ACA GCT CAC CTC AAT AAG

GCC AGC CTG CAT GTT TCC CTG CAC ATC AGA GAC TCC CAG CCC AGT

GAC TCC GCT CTC TAC TTC TGT GCA AGC TAA CTC CGC TCT CTA CTT

CTG TGC AGC TAA CAC GGG TTA CCA GAA CTT CTA TTT TGG GAA AGG

AAC AAG TTT GAC TGT CAT TCC AAG TAA GTA

Figure 6.3 Nucleotide sequence of the cDNA encoding the 9.13 TCR a chain. Sequencing analysis revealed that the 9.13 T cell hybridoma utilizes an α chain of the Vα1.1 family rearranged with Jα49 (bold sequence). Underlined sequence represents the beginning of the constant region Cα.

6.3.3 *Characterization of the β TCR chain gene expressed on the 9.13 T-cell hybridoma.*

It has been previously found that the β chain expressed by the 9.13 T cell hybridoma belongs to Vβ4 family (Rao et al. 1997) but the DJ region was unknown. Primers from the beginning of the leader sequence of the published Vβ4 gene and also from the 3′ of the constant β chain were designed to amplify cDNA from the 9.13 clone, as described in Materials and Methods. Upon PCR amplification, a single band of the expected size (950 bp -Figure 6.4) for the β chain gene was observed. The amplified β chain gene was gel purified and ligated into the pcDNA3.1/zeo$^+$ mammalian vector. The ligated product was used to transform competent cells and plasmid DNA from the resulting colonies was isolated and sequenced. From the sequencing results we confirmed the utilization of the Vβ4 gene rearranged with the D2.1-J2.1 gene segments (Figure 6.5). Additions of N nucleotides were also observed without altering the reading frame of the β chain gene (Figure 6.5).

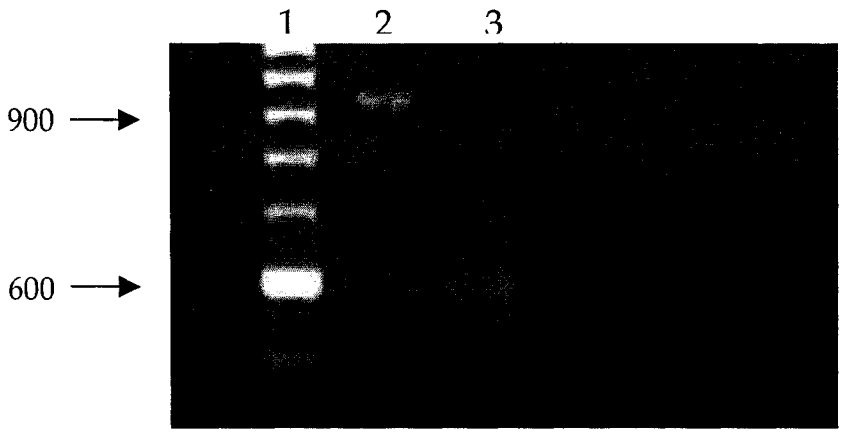


Figure 6.4 Amplification of the entire TCR-β chain gene from the 9.13 T cell hybridoma clone by RT-PCR.

RNA was extracted from the T-cell clone, and was reverse transcribed in cDNA. RT-PCR was performed using gene specific primers as described in Materials and Methods. A single band of the expected size (lane 2) contains the β chain gene. Amplification of the GADPH gene was used as positive contol (lane 3). PCR products were visualized in 1.5% agarose gel. 100bp DNA ladder is shown in lane 1.

ATG GGC TCC ATT TTC CTC AGT TGC CTG GCC GTT TGT CTC CTG GTG

GCA GGT AGT CCT AGT CTT GGC CTA TAC ACC TTT GTA TTT TAC ATA

GAA CAT CCT TCT ACA ATT ATC TTG AGG ATT AAA CTT GTT TTC TTA

TTT CCA CAG GTC CAG TCG ACC CGA AAA TTA TCC AGA AAC CAA AAT

ATC TGG TGG CAG TCA CAG GGA GCG AAA AAA TCC TGA TAT GCG AAC

AGT ATC TAG GCC ACA ATG CTA TGT ATT GGT ATA GAC AAA GTG CTA

AGA AGC CTC TAG AGT TCA TGT TTT CCT ACA GCT ATC AAA AAC TTA

TGG ACA ATC AGA CTG CCT CAA GTC GCT TCC AAC CTC AAA GTT CAA

AGA AAA ACC ATT TAG ACC TTC AGA TCA CAG CTC TAA AGC CTG ATG

ACT CGG CCA CAT ACT TCT GTG CCA GCA GCC AAG AC*T GGG CGT* CTT

CGT CTG AGC AGT TCT TCG GAC CAG GGA CAC GAC TCA CCG TCC TAG

GTA AGA

Figure 6.5 Nucleotide sequence of the cDNA encoding the 9.13 TCR b chain.

Bold sequence represents the leader sequence of the β chain rearranged with the Vβ4 -Dβ2.1 (italic sequence)-Jβ2.1 (bold sequence)-Cβ (underlined). The addition of N nucleotides is shown (double underlined).

6.3.4 *High levels of Vβ4 chain expression on the surface of the 9.13 T-cell hybridoma.*

To examine if 9.13 T cell hybridoma consists of one T cell population we looked at the level of Vβ4 expression on the surface of these cells. We stained the cells with mAbs specific for the Vβ4 chain as well as anti-TCR and anti-CD3 mAbs and analysed them with FACS analysis. Since Vα1.1-specific mAb was not commercially available we only assessed β chain expression. Upon staining with anti-CD4 and anti-TCR, 77% were stained double positive, and 76% were found to express the Vβ4 chain indicating that almost 100% of the T cells were expressing the β4 family (Figure 6.6). No expression of the Vβ4 chain was observed with the myeloma cell line BW5147$\alpha^-\beta^-$ that was used for the fusion during the generation of the T-cell clone (Figure 6.6).

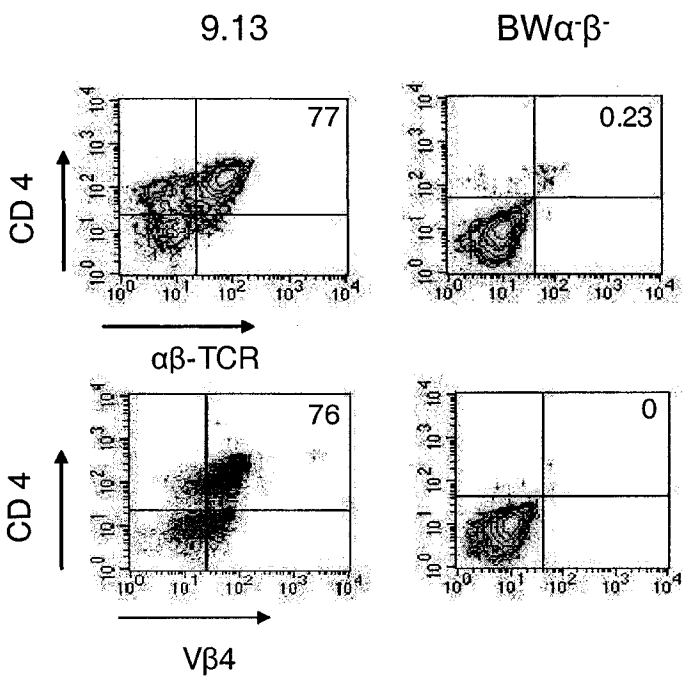


Figure 6.6 Phenotype of T cells from the 9.13 T cell hybridoma.

Cells were surface stained with anti-CD4 (y axis) and anti-αβTCR (x axis) or with anti-CD4 and anti-Vβ4 (x axis) specific monoclonal antibodies. As contol, cells from the BWα-β- cell line were used. The percentages of double positive cells are shown in the upper right boxes.

6.3.5 *Identification of the appropriate pTα and pTβ cassette vectors upon digestion with combination of restriction enzymes*

In order to generate the TCR transgenic mice we used the pTα and pTβ cassette vectors (Kouskoff et al. 1995), for expression of the α and β TCR chains respectively. To identify vectors with the correct profile, plasmid DNA was purified from several colonies that were obtained upon transformation of XL-10 GOLD competent cells with pTα and pTβ. Subsequently the plasmids were digested with different combinations of restriction enzymes: the pTα vector was digested with BamHI, SalI and SalI/NotI, and the pTβ vector with EcoRI, KpnI and XhoI/SacII. The expected fragments were calculated according to the published map of each vector (Kouskoff et al. 1995). Specifically, digestion of the pTα vector with BamHI resulted in 5 bands at: 2.3 Kb, 3.0 Kb, 3.3 Kb, 5.2 Kb and 8.5 Kb whereas digestion with the SalI and SalI/NotI resulted in 2 fragments at 5.2 Kb and 17.1 Kb or 3 fragments at 2.8 Kb, 5.2 Kb and 14.3 Kb respectively (Figure 6.7). Similarly, digestion of pTβ with EcoRI resulted in one band at 0.9 Kb, one at 2.2 Kb, two bands between 3-4 Kb and a fifth band at 10 Kb. Upon digestion of pTβ with KpnI a band at 17.6 Kb and one at 2.9 Kb were obtained, whereas with XhoI and SacII one band at 0.6 Kb and one at 19.9 Kb were found (Figure 6.7). Vectors with the correct profile were further used for generation of the α and β gene constructs.

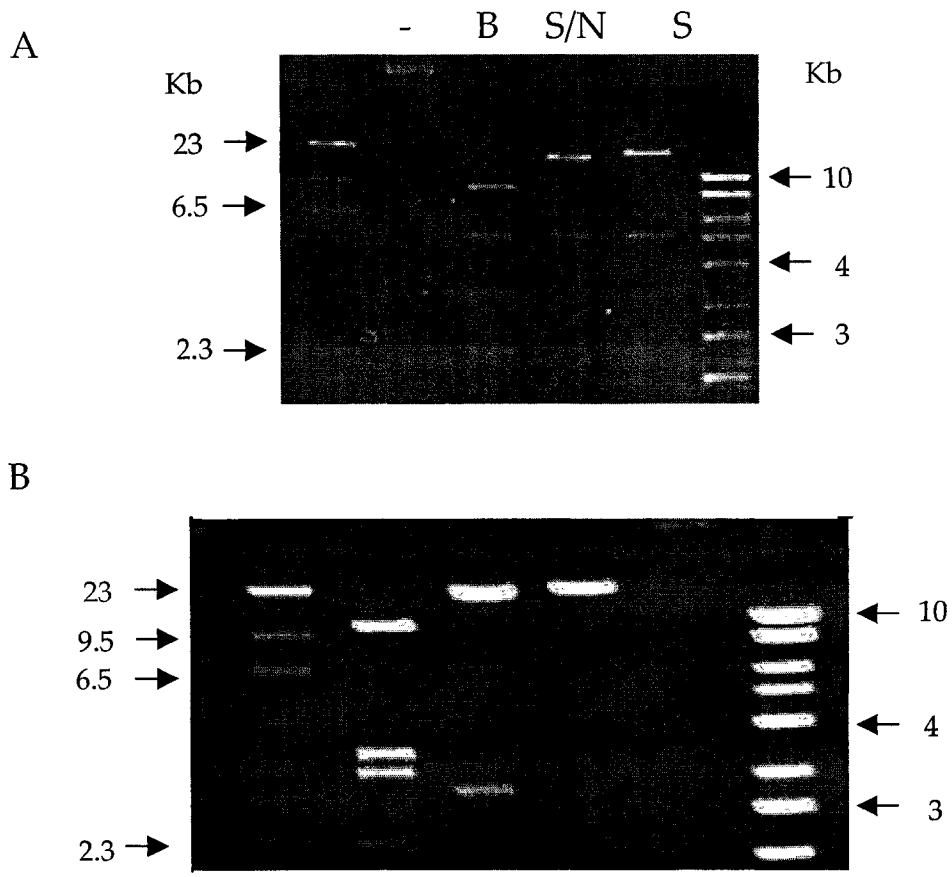


Figure 6.7 Restriction enzyme analysis of the original pTα (A) and pTβ (B) cassette vectors.

The pTα vector was digested with BamHI (B), SalI (S) or SalI and NotI (S/N). The pTβ cassette was digested with EcoRI (E), KpnI (K) or XmaI and SacII (X/S). Undigested vectors are also shown (-). The λDNA/HindII ladder and the 1 Kb DNA ladder were used to calculate the size of the digested fragments. Electrophoresis in 0.8% agarose gel was performed at 70V for 1h 45min.

6.3.6 *The VJα and VDJβ gene segments were amplified from genomic DNA and cloned into the pTα and pTβ cassette vectors respectively.*

To generate the microinjection constructs, genomic DNA was isolated from the 9.13 T-cell hybridoma and amplification of the LVJα (~ 650 bp) and LVDJβ (~ 570 bp) segments was performed as described in Materials and Methods. The complete LVJα segment was amplified in two sequential rounds of PCR (Figure 6.8) and the genomic fragment of the second round was inserted into the pCR4-TOPO. Plasmid DNA that contained the α chain gene was sequenced in order to ensure fidelity of the α chain gene segment. Genomic LVJα fragment that was in frame was excised from the pCR4-TOPO upon digestion with XmaI/NotI and its quantity was measured with the 2-Log DNA ladder. The VDJβ segment was amplified using only one round of PCR (Figure 6.8) and was digested with Xho-I/Apa-I in order to be ligated into the pcDNA3.1/zeo$^+$ vector. Genomic LVDJβ fragment that was in frame was released upon digestion of pcDNA3.1/zeo$^+$ vector with XhoI/SacII. The digested genomic LVJα and LVDJβ fragments were ligated into similarly digested pTα and pTβ vectors respectivelly, using equimolar quantities, as described in Materials and Methods. The pTα/LVJα and pTβ/LVDJβ cassettes with the appropriate profile were quantified, and linearized by excising the prokaryotic sequences upon digestion

of the pTα cassetteswith SalI, and the pTβ cassettewith Kpn I. The respective 17Kb and 17.5Kb fragments were gel-purified, and quantified prior to microinjection.

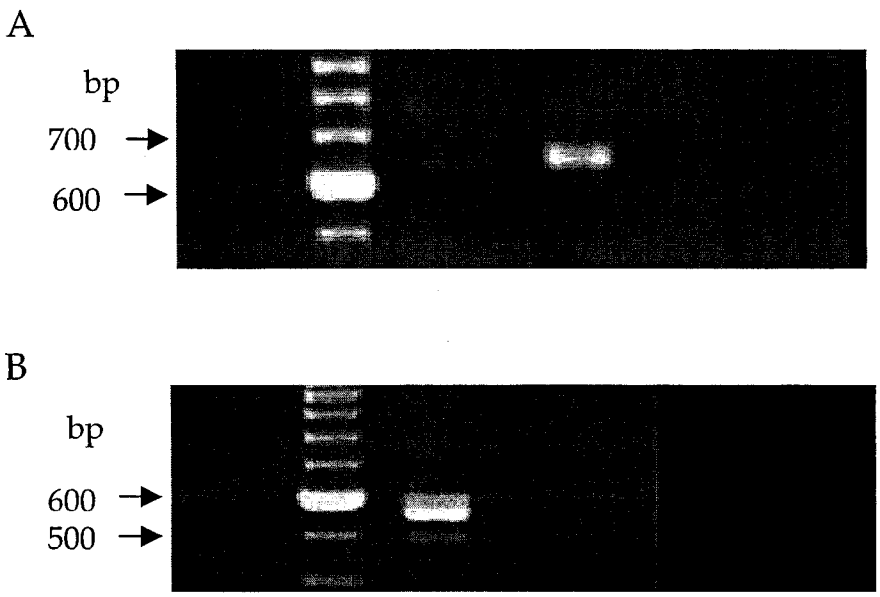


Figure 6.8 Amplification of the genomic α and β gene segments.

The VαJα (650 bp) gene segment (A) and the VDJβ (570 bp) gene segment (B) were amplified from the 9.13 T cell hybridoma genomic DNA. The α gene was amplified in two rounds of PCR as described in Materials and Methods. Amplification was performed by PCR in 35 cycles and the products were subjected to agarose electrophoresis (1.5% agarose gel). The 100 bp DNA ladder is shown.

6.3.7 *Successful integration of the 9.13 T cell receptor α and β chain genes.*

To examine if the TCR α and β transgenes successfully integrated into the germline of founder mice, DNA was isolated from mouse tails and subjected to PCR using primers specific for the 9.13 TCR α and β chain genes. Six mice (defined as 2BC.F1-2BC.F6) were screened at the 4th week of age. Using the gene specific primers, the expected size for tha 9.13 α chain is 593 bp whereas for the 9.13 β chain is 591 bp. Two out of six mice (2BC.F2 and 2BC.F3) were identified to contain both 9.13 TCR α and β chain genes (Figure 6.9). Internal control primers which amplify TCRδ exon1 gene were also used in each PCR reaction in order to confirm the presence of sufficient amount of DNA. The expected size of the TCRδ exon 1 amplified gene is 206 bp (Figure 6.9).

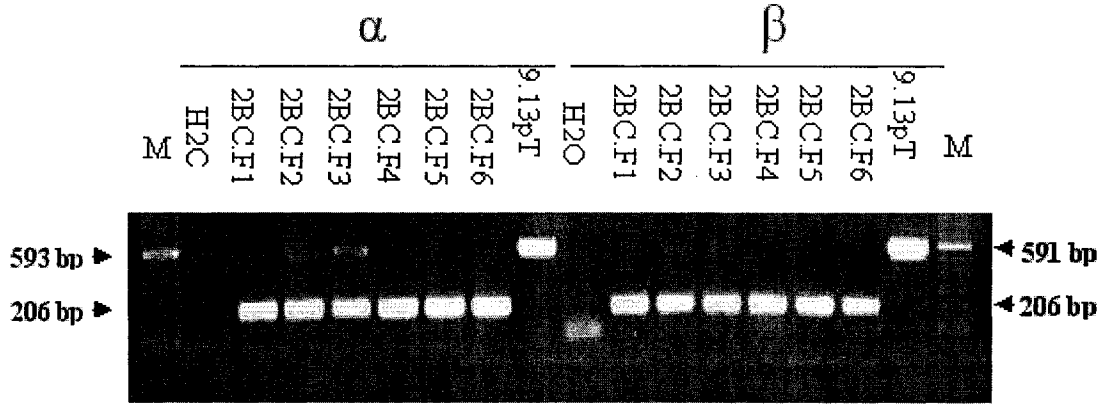


Figure 6.9 PCR demonstration for the identification of p2496-specific TCR transgenic mice.

Mice that have incorporated the 9.13 α and β TCR chains were identified by PCR. DNA was extracted from mice tail and a duplex PCR was performed as described at Materials and Methods. Primers specific for the α, β as well as the TCRδ exon 1 gene were used. In each run, a no-template control and a plasmid control were included. Final PCR products were analysed by agarose gel eletrophoresis (Courtesy by Jinguo Wang).

6.3.8 *9.13 TCR transgenic T cells specifically activates in response to p2496 in vitro.*

In order to examine if the 9.13 TCR transgenes were expressed on the surface of the TCR transgenic mice, spleens were isolated from founder mice and single cell suspensions prepared as described in Materials and Methods. As control wild type SJL mice were used. The cells were tested for their ability to proliferate in the presence of the p2496 Tg peptide or OVA antigen as control. Only cells from transgenic mice proliferated in the presence of p2496 Tg peptide. Proliferation was antigen specific since was not observed in the presence of OVA (Figure 6.10). The above data indicate the successful generation of transgenic mice that express a TCR specific for the p2496 Tg peptide.

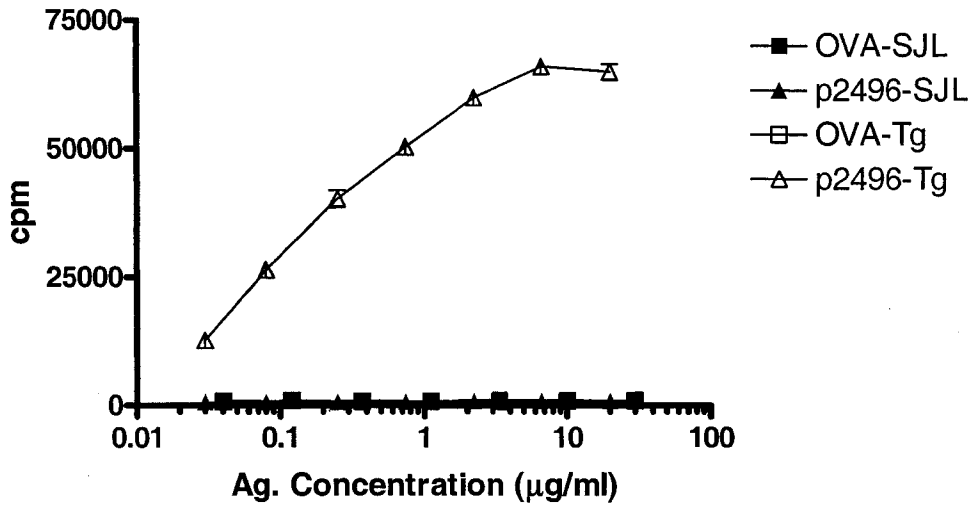


Figure 6.10 In vitro T cell proliferation of p2496-specific TCR transgenic mice

Splenocytes were isolated either from 9.13 TCR transgenic or SJL mice and were adjusted at $4x10^6$ cells/ml. Cells were cultures (200 μl/well) in the presence of p2496 peptide or OVA antigen as control. Followed 56h incubation, 1μCi ^3H TdR was added to each well for the last 16h. Cells were harvested and cpm counting with a micro-scintillation counter was performed as described in Materials and Methods. (Courtesy by Jinguo Wang)

6.4 Discussion

In this study, we focused on the generation of transgenic mice, that carry a TCR specific for the cryptic p2496 Tg peptide, on the SJL background. These mice will provide an abundant source of naïve p2496-specific T cells that will be used to answer questions on the activation of the autoreactive T cells and their role in EAT pathogenesis. In addition, immmunoregulation studies will be performed with the aim to identify protocols to suppress the pathogenic potential of the p2496-specific T cells.

For the expression of the 9.13 TCR α and β chains we utilized the pTα and pTβ cassette vectors respectively. Both vectors have been engineered to contain natural TCR α and β promoter and enchancer elements in order to ensure specific expression of the α and β on the T cell surface. One potential problem working with such large vectors (~20 Kd each) was that during cloning procedures several short plasmids could be obtained due to false recombination events. Therefore, competent cells were transformed with either pTα or pTβ vectors and colonies were screened with combinations of restriction enzymes (as described in paragraph 6.3.5) in order to identify cassettes with the correct profile. Upon screening, 10-20% of the vectors were found to express the expected profile and were further used to prepare the 9.13 constructs. Screening

for cassettes with the appropriate profile was also performed after the generation of 9.13 pTα and pTβ constructs.

Most of the TCR transgenic mice that have been described in the literature do not develop autoimmunity spontaneously (Alderuccio et al. 2000;Goverman et al. 1993;Lafaille et al. 1994), and this finding has been attributed in part to the presence of regulatory cell populations in the periphery of those mice (Olivares-Villagomez et al. 1998;Van de and Tonegawa 1998). For example, when MBP-specific TCR transgenic mice were crossed with RAG-deficient mice (to remove the non-transgenic lymphocytes), 100% of transgenic mice develop spontaneously EAE (Lafaille et al. 1994). In contrast to the above findings, all transgenic mice expressing a TCR derived from a gastritogenic CD4+ T cell clone developed gastritis spontaneously without need for reducing the endogenous T cell repertoire (McHugh et al. 2001). So far the 9.13 TCR transgenic mice have been backcrossed for five generations to SJL background and are housed in a pathogen free environment. No spontaneous development of EAT have been noticed in these mice indicating lack of sufficient presentation of the p2496 peptide in vivo. Additionally, p2496-specific CD4+CD25+ T cells could exist in 9.13 TCR transgenic mice with the ability to suppress the activation of p2496-specific effector T cells. Immunization of transgenic mice with p2496 in CFA induced robust activation of p2496-specific T cells and massive infiltration of the

thyroid gland was observed 10 days post immunization (experiments performed by Jinguo Wang)

The p2496-specific TCR transgenic mice will provide useful information on the pathogenic mechanisms underlying EAT development and will allow the testing of various regulatory protocols aimed at the amelioration of the disease.

Summary

Experimental autoimmune thyroiditis induced by Tg and adjuvant in mice is considered to be a model for Hashimoto's thyroiditis in man. In this study, we did an algorithm-based search to map Tg peptides containing A^k-binding motifs and we identified five Tg peptides that were pathogenic in CBA/J mice (Chapter 3). None of the five peptides encompass dominant epitopes, but nevertheless, they will be very useful in the study of mechanisms that promote their generation within APC, thus assigning to them a potential role during the autoimmune cascade. One of the five peptides, p2596, was tested and found to be pathogenic in several H-2^k strains but not in mice of b, d, s or q haplotypes (Chapter 4).

The experiments in Chapter 5 describe that TNF-α-treated dendritic cells pulsed with Tg induce a Tg-specific CD4+CD25+ T cell population (Treg) that suppresses Tg-specific immune responses and Tg-induced EAT. The CD4+CD25+ Treg cells secrete IL-10 upon activation with Tg in vitro but transwell experiments indicated that soluble mediators are not sufficient to mediate their suppressogenic function; instead, cell-to-cell contact between these regulatory cells and effector cells is required. These data create a new impetus to

study the mechanisms underlying the induction or effector function of Tregs as well as to identify molecules that are engaged during this process. Additionally, we can investigate whether Tg-specific CD4+CD25+ T cells can revert established EAT, significant question from a clinical point of view.

Finally, Chapter 6 describes the effort to generate TCR - transgenic mice whose T cells are specific for the cryptic p2496 Tg epitope. These mice will enable the isolation of large numbers of p2496-specific naïve T cells and they will be extremely useful for investigating parameters that can influence: a) the generation and presentation of the p2596 cryptic epitope on APC and b) the subsequent activation of p2596-specific T cells leading to EAT development. Moreover, the availability of naïve p2596-specific TCR transgenic cells may allow us to establish protocols under which they can become Treg with the ability to suppress the development of autoimmunity .

Chapter 7

FUTURE DIRECTIONS

The identification of immunodominant T cell epitopes in autoantigens has been critical in the study of organ-specific autoimmunity. This is because induction of autoimmune responses has been directed to dominant T cell determinants. Therefore, delineation of immunodominant epitopes will allow the study of immune effector mechanisms that lead to disease development as well as the design of antigen specific therapeutic strategies. When I started this study, five pathogenic Tg epitopes had been mapped and classified as non-dominant. Therefore, the current work was initiated with the aim to identify immunodominant epitope(s) within the Tg molecule. Using an algorith approach we discovered five new pathogenic Tg peptides in CBA mice. However, none of the five Tg peptides encompass a dominant epitope. Although, knowledge of an immunodominant Tg epitope is of great interest, identification of cryptic Tg determinants however, generates new questions on parameters that influence their generation in vivo and allow the investigation of their role in disease immunoregulation (Carayanniotis 2003;Moudgil and Sercarz 2005).

Hypothesis 1: cryptic epitopes are involved in EAT pathogenesis due to epitope spreading.

The phenomenon of epitope spreading was first described in EAE (Lehmann et al. 1992;Lehmann et al. 1993;Moudgil and Sercarz 1994). It was shown that EAE mediated by intact myelin basic protein (MBP) or the immunodominant epitope (Ac 1-11) can spread (within 40 days post immunization) both inter-molecularly to other autoantigens and intra-molecularly to other non-dominant determinants (Lehmann et al. 1992). To test "determinant spreading" in EAT, susceptible mice will be immunized with Tg in CFA. Spleen and thyroid-draining lymph node cells (LNC) will be isolated on day 10, 20, 30 and 40 after immunization and will be examined for their proliferative responses against a panel of cryptic Tg peptides (those identified previously (Carayanniotis and Kong 2000) and in this study (Verginis et al. 2002) as well as intact Tg in vitro. According to our hypothesis, we should observe significant proliferation of Tg-primed splenocytes or thyroid-draining LNC against some of the cryptic peptides tested, beyond the 20 days post Tg immunization. This result will indicate the importance of the cryptic epitopes in the evolution of Tg-induced EAT. In addition, it will significantly aid future studies on the immunoregulation of EAT studies since one should consider that specific immunotherapy would directed not only against the dominant epitopes

of an autoantigen but also against epitopes that appear during the progression of the disease.

In case that no reactivity occurs against the Tg peptides tested it could be possible that the autoimmune response is directed against other cryptic Tg pathogenic peptides that have not been identified or against epitopes from other thyroid autoantigens such as TPA and TSHR (intramolecular spreading). Additionally, cryptic peptides could become dominant intrathyroidically after disease progression and therefore will not be able to detect them in the draining lymph nodes.

Hypothesis 2: Tg cryptic epitopes must be generated and expressed by intra-thyroidal APC to exert their immunopathogenic effect.

To test this hypothesis, we should first establish primary thyrocyte cultures, as previously described (Jeker et al. 1999), or isolate thyroid-derived dendritic cells that originate from normal EAT susceptible strain of mice. Thyrocytes or DCs will be cultured in the presence of IFN-γ to up regulate the expression of MHC class II molecules and the co-stimulatory molecules that are essential for T cell activation. In parallel, we will generate T cell hybridomas or T

cell clones specific for the cryptic Tg pathogenic peptides. These clones will be cultured in the presence of the thyrocyte or DC cultures in the absence or presence (positive control) of exogenously added peptide. According to our hypothesis, significant proliferation of peptide-specific T cell clones will occur in the absence of the respective Tg peptide and that will indicate the intra-thyroidal generation of the cryptic Tg peptides. If peptide-specific proliferation does not occur, it could be due to not sufficient presentation of peptide in the thyrocytes or DCs in vitro. In this case, only thyrocytes and DCs that pulsed with exogenously added peptide should activate the T cells clones.

Hypothesis 3: Cryptic Tg peptides are involved in mouse model of spontaneous thyroiditis development.

One of the most studied animal models that develop SAT is the NOD.2h4 mouse (Braley-Mullen et al. 1999). The NOD.2h4 mice develop 100% thyroiditis when they drink 0.05 NaI (Braley-Mullen et al. 1999;Rasooly et al. 1996). To test the above hypothesis, spleens and thyroid-draining lymph nodes will be collected from NOD.2h4 mice at the time or before of disease initiation and during disease progression. As negative control, NOD. SWR (H-2q) (Braley-Mullen et al. 1999) will also be used following the same treatment. Isolated cells will be tested for proliferative response and cytokine secretion in the presence of

the known pathogenic Tg peptides (mentioned above). According to our hypothesis, only T cells isolated from the NOD.2h4 mice, should proliferate in response to the Tg peptides and should secrete significant amounts of IL-2 and/or IFN-γ. Identification of pathogenic epitope(s) in NOD.2h4 mice will allow us to investigate immunoregulation mechanisms (such as induction of peptide-specific suppressor cells) in a spontaneously induced thyroiditis model.

It has been shown that regulatory cells participate in the maintenance of tolerance (Sakaguchi et al. 2001;Shevach 2000). My present work demonstrates that DCs with semi-mature phenotype are able to induce $CD4^+CD25^+$ T cells with the ability to suppress Tg-induced EAT. Several questions remain to be answered on the induction, function and specificity of the Treg cells as well as how tolerogenic DCs can be formed under physiological conditions.

Hypothesis 1: Upon endocytosis of apoptotic thyrocytes, immature DCs adapt a "semi-mature" phenotype similar to that induced in the presence of the TNF-α in vitro.

There is increasing evidence that immature DCs in steady state can traffic through tissues and pick up cells that die through apoptosis due to normal cell turn over (Albert et al. 1998;Huang et al. 2000;Inaba et al. 1998). To test the above hypothesis we will generate thyrocyte cultures (as described above) and apoptosis will be triggered by UV irradiation (Sauter et al. 2000). Immature DCs will be prepared from femurs and tibias of CBA mice as described in Materials and Methods and will be cultured with the apoptotic thyrocytes at cell equivalent ratios of 1:1, 2:1 and 1:2 for 3h at 4°C or 37°C. In order to determine if the apoptotic thyrocytes are efficiently phagocytosed by the DCs, both cell populations will be labeled with different fluorochromes (such as PKH26-GL and PKH67-GL red and green fluorescents respectively- Sigma) before incubation. Phagocytosis will be defined by the percentage of the double positive cells using FACS analysis. As controls, non-apoptotic thyrocytes will be used. The DCs will be washed to remove the non-endocytosed thyrocytes and will be examined for the surface phenotype as well as the cytokines that they secrete. According to our hypothesis, we expect only DCs that have up taken apoptotic thyrocytes to express high levels of MHC class II and costimulatory molecules and to secrete low levels of proinflammatory cytokines such as IL-12, IL-6 and IL-1β. The phenotype of DCs/apoptotic thyrocytes will be compared with the phenotype of

TNF-α-treated DCs that have been shown to possess tolerogenic properties (Menges et al. 2002;Verginis et al. 2005).

Hypothesis 2: DCs that have internalized apoptotic thyrocytes, are able to induce tolerance to thyroid antigens through the generation of antigen-specific $CD4^+CD25^+$ T cells.

Following the rationale of the previous hypothesis, we will now examine if DCs that have captured apoptotic thyrocytes can induce tolerance. The DCs/thyrocytes will be prepared as described above, and will be injected into CBA mice intravenously at day 1, 3 and 5. Unpulsed DCs or DCs pulsed with non-apoptotic thyrocytes will be used as controls. On day 7, mice will be challenged with Tg or thyroid extract (peptides derived from thyroid antigens such as TPO could be also presented by DCs) in CFA. EAT will be assessed on day 21 after the antigenic challenge. If our hypothesis is correct, we expect to see a significant decrease of the EAT incidence in the group of mice that received DC/apoptotic thyrocytes, compared with the control groups. In parallel, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations will be isolated (21 days post immunization) from mice that have been injected with DCs but did not receive antigenic challenge. Both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations will be

tested for their function in cultures with the effector T cells isolated from Tg- or thyroid extract-immunized syngeneic mice. According to our hypothesis, only $CD4^+CD25^+$ T cells from mice challenged with DC/apoptotic thyrocytes should suppress the effector T cells.

In case that the data obtained from these experiments do not support the hypothesis it could be due to presence of other antigens that are contained in the apoptotic thyrocytes and compete with the Tg peptides for presentation by the DCs.

Hypothesis 3: Suppression of Tg-specific autoreactive T cells, mediated by the CD4+CD25+ T cells generated by Tg/DCs, is antigen-specific and is not due to bystander effect.

According to my results, the activation and/or expansion of Treg cells by Tg/DC, but not OVA/DC, indicates that the phenomenon is Tg-specific. To examine if the suppressor effector function of the Treg is antigen specific, we will utilize Tg pathogenic peptides as antigens. Several studies have demonstrated the presence of Treg cells specific for dominant self-peptides (Cozzo et al. 2003;Mukherjee et al. 2003;Picca and Caton 2005;Yu et al. 2005). To test our hypothesis, TNF-α-treated DCs will be pulsed with p2596 Tg peptide (randomly

selected) and will be injected i.v. into CBA mice on day 1, 3 and 5. On day 7, mice will be s.c. immunized with p2596 or p306 in CFA. Control mice will receive PBS instead of the DC injections. Twenty-one days later, mice will be sacrificed for assessment of thyroiditis. A decrease in EAT incidence is expected in mice that receive DC/p2596 and immunized with p2596 but not p306 Tg peptide, as compared to the control group. This finding will indicate that DC/p2596 activate p2596-specific Treg that suppress p2596-specific T cell responses. Additional evidence supporting our findings will be obtained by in vitro experiments. $CD4^+CD25^+$ T cells will be isolated from splenocytes of mice challenged with DC/p2596 (3x as above) and will be used as suppressors. Effector cells will be isolated from the lymph nodes of mice that have been challenged with p2596 or p306 Tg peptide. Effector cells will be cultured in the presence or absence (control) of the suppressor cells (at equimolar ratio) as well as syngeneic APC and the respective peptide. If our hypothesis is correct, significant decrease in the proliferation of the p2596-effector but not of the p306-effector T cells should be demonstrated when compared with cell proliferation in control cultures.

Hypothesis 4: Tg-specific CD4+CD25+ T cells can reverse established EAT.

To test this hypothesis, we will first isolate CD4+CD25+ T cells from DC/Tg- and DC/OVA-challenged CBA mice, as described above. Syngeneic mice will be immunized with 100 μg Tg in CFA. Three weeks later, some mice will be sacrificed to assess EAT. Based on our results, at that time point mice develop EAT with mean I.I. of 2.2. The remaining mice will be boosted with 50 μg Tg in IFA and will also be challenged i.p. with CD4+CD25+ T cells from DC/Tg or DC/OVA-challenged mice. Control group will receive PBS instead of CD4+CD25+ T cells. Two weeks later all mice will be sacrificed and thyroids will be assessed for EAT development. According to our hypothesis, mice that received CD4+CD25+ Treg cells from DC/Tg- but not DC/OVA-treated mice should have decrease EAT incidence compared with the control group.

Finally, the TCR transgenic line specific for the p2496 Tg peptide, described in Chapter 6, provides an invaluable tool to study mechanisms that are involved in the precipitation or amelioration of EAT. Although in our TCR-transgenic model high frequency of p2496 Tg peptide-specific T cells will be obtained it is difficult to predict if mice will develop or not EAT. Therefore, we hypothesize that the TCR-transgenic mice will not develop spontaneous EAT.

Hypothesis 1: In vivo presentation of the p2496 by DC results in the activation of p2496-specific Th1 cells and induction of EAT.

To test this hypothesis, BM-DC will be isolated from SJL mice (as described above) and will be cultured in the presence of LPS for 24h in order to mature. Maturation of DCs will be examined by flow cytometry for upregulation of MHC class II and B7.1/B7.2 expression by sandwich ELISA for secretion of IL-12, IL-1 and IL-6. In the next step, DCs will be pulsed with p2496 for 6h and will be adoptively transferred i.p. into TCR transgenic mice specific for the p2496. Control mice will receive unpulsed DCs or DC pulsed with another pathogenic Tg peptide. Twenty-one days after the DC/peptide transfer, mice will be sacrificed and thyroids will be collected and assessed for EAT. According to our hypothesis, mice received p2496-pulsed DCs will develop EAT in contrast to control mice, which should be free of disease. Based on our results, we can conduct a time-kinetic of the EAT induction by collecting thyroids at different times after the DC transfer. We can also adoptively transfer peptide-pulsed DC for three consecutive days to achieve higher incidence of EAT. Priming of p2496-specific Th1 will be examined by T cell proliferation and cytokine release of cells isolated from the spleen of the TCR transgenic mice 10-14 days after DC transfer.

These experiments will allow us to develop an EAT induction model without the use of adjuvants or in vitro T cell activation.

Hypothesis 2: Processing of I-Tg by DCs leads to generation of p2496 and activation of p2496-specific T cells in vivo.

Previous work in our lab, demonstrated that processing of I-Tg but not Tg by DCs results in the generation of the cryptic p2496 Tg peptide (Dai et al. 2002). Using the p2496-specific TCR transgenic mice, we can assess the above hypothesis directly in vivo. DCs will be pulsed with I-Tg , Tg or the p2496 Tg peptide and will subsequently treated with LPS to mature. Unpulsed mature DCs will be used as controls. Antigen-bearing or unpulsed DCs will be adoptively transferred i.p. into the TCR transgenic mice. Priming of $CD4^+$ T cells will be monitored 12-14 days after cell transfer. Splenocytes will be isolated and cultured in the presence of I-Tg, Tg, p2496 and control peptide. According to our hypothesis, T cells isolated from mice that challenged with DC/I-Tg and DC/p2496 and not with DC/Tg should proliferate and secrete IL-2 and IFN-γ against the respective antigens in vitro. Moreover, transgenic T cells from DC/I-Tg- or DC/p2496-challenged mice will proliferate in the presence of p2496 and I-Tg respectively. Additional evidence to support our hypothesis will be obtained

from the histology of the thyroid gland of the above groups of mice. Specifically, we expect to observe induction of EAT in mice that received DC/I-Tg and DC/p2496.

Hypothesis 3: presentation of p2496 by smDCs can lead to the expansion of p2496-specific CD4+CD25+ Treg cells.

To test our hypothesis, we will prepare smDCs (as described above) and pulse them with p2496 peptide. The smDC/p2496 cells will be adoptively transferred on day 1, 3 and 5 into the p2496-specific TCR transgenic mice. Control mice will be challenged with unpulsed DCs at the same time points. The mice will be divided in three groups, which will be sacrificed on days 10, 15 and 20 post-transfer, and splenocytes will be collected. CD4+CD25+ and CD4+CD25- T cells will be prepared and will be tested in vitro by proliferative assays and cytokine production in the presence of p2496 or a control Tg peptide. Only CD4+CD25- T cells should strongly proliferate in the presence of p2496 in vitro. To support our hypothesis, we will conduct a mixing experiment where CD4+CD25+ T cells should suppress the proliferation of syngeneic p2496-specific CD4+CD25- T cells. If the results support our hypothesis, we will seek additional confirmation by transferring p2496-specific CD4+CD25+ T cells (generated as

above) into syngeneic mice that have been challenged with p2496 in CFA prior to or post T cell transfer and monitor EAT. Control mice will receive CD4+CD25+ T cells isolated from mice challenged with DCs alone or with DC that present another Tg pathogenic peptide. We expect to observe amelioration of EAT only in mice that received p2496-specific CD4+CD25+ T cells.

If CD4+CD25+ T cells cannot inhibit EAT induction, despite their suppressive activity in vitro, it might be due to not sufficient cell numbers that were transferred. Therefore, we will perform kinetics to determine the adequate number of CD4+CD25+ T cells that have to be transfered in CBA mice to ameliorate EAT.

Hypothesis 4: FoxP3-transduced p2496-specific T cells can suppress EAT.

Recent studies have demonstrated that expression of FoxP3 on antigen-specific T cells can revert them to regulatory T cells with the potential to suppress autoimmunity (Fontenot et al. 2003;Jaeckel et al. 2005). To test the above hypothesis, FoxP3 mRNA will be isolated from SJL mice and cDNA will be amplified using gene-specific primers with incorporated restriction sites. The amplified cDNA fragment will be cloned into a retroviral vector (Moloney murine leukemia virus MMLV) as previously described (Fontenot et al.

2003;Loser et al. 2005). Empty retroviral vector will be used as control. Retrovirus-containing supernatants (encoding FoxP3 or "empty") will be collected upon transfection of a packaging cell line (commercial available) with retrovirus encoding plasmid DNA. CD4+CD25- T cells isolated from the spleens of either from p2496-specific TCR transgenic mice or naïve SJL mice will be infected with high titers of FoxP3-containing or "empty" retrovirus using centrifugation as described (Jaeckel et al. 2005). Sufficient expression of FoxP3 on infected cells will be monitored by FACS analysis. To test our hypothesis, p2496-specific CD4+CD25- T cells (effectors) isolated from TCR transgenic mice will be cultured with p2496 peptide and syngeneic APC as well as equal numbers of CD4+CD25- T cells expressing or not FoxP3 (regulators). Only effector cells cultured with CD4+CD25- T cells infected with the "empty" retroviral vector should proliferate and secrete IL-2 and IFN-γ in response to p2496 in vitro whereas FoxP3-expressing p2496-specific CD4+CD25- T cells should suppress the proliferation of the p2496-specific effector cells. In addition, retroviraly-infected CD4+CD25- T cells will be adoptively transferred (3X) to p2496 TCR transgenic mice on day 0 and on day 1 mice will be immunized with p2496 in CFA. Another group of mice will receive PBS and will be used as additional control. If our hypothesis is correct, TCR transgenic mice that received FoxP3-expressing p2496-expressing CD4+CD25- T cells will shown decreased incidence of EAT compared

with control groups. Additional evidence for the suppressogenic properties of FoxP3-expressing antigen-specific CD4+CD25- T cells could have a therapeutic potential in patients with autoimmune thyroiditis.

Reference List

Ajjan RA, Watson PF, McIntosh RS, Weetman AP (1996) Intrathyroidal cytokine gene expression in Hashimoto's thyroiditis. Clin. Exp. Immunol. 105:523-8

Akbari O, DeKruyff RH, Umetsu DT (2001) Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat. Immunol. 2:725-31

Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N (1998) Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J. Exp. Med. 188:1359-68

Alderuccio F, Cataldo V, van Driel IR, Gleeson PA, Toh BH (2000) Tolerance and autoimmunity to a gastritogenic peptide in TCR transgenic mice. Int. Immunol. 12:343-52

Alimi E, Huang S, Brazillet MP, Charreire J (1998a) Experimental autoimmune thyroiditis (EAT) in mice lacking the IFN-gamma receptor gene. Eur. J. Immunol. 28:201-8

Alimi E, Huang S, Brazillet MP, Charreire J (1998b) Experimental autoimmune thyroiditis (EAT) in mice lacking the IFN-gamma receptor gene. Eur. J. Immunol. 28:201-8

Allen PM, Matsueda GR, Evans RJ, Dunbar JB, Jr., Marshall GR, Unanue ER (1987) Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. Nature 327:713-5

Allen PM, Matsueda GR, Haber E, Unanue ER (1985) Specificity of the T cell receptor: two different determinants are generated by the same peptide and the I-Ak molecule. J. Immunol. 135:368-73

Allen PM, Strydom DJ, Unanue ER (1984) Processing of lysozyme by macrophages: identification of the determinant recognized by two T-cell hybridomas. Proc. Natl. Acad. Sci. U. S. A 81:2489-93

Altuvia Y, Berzofsky JA, Rosenfeld R, Margalit H (1994) Sequence features that correlate with MHC restriction. Mol. Immunol. 31:1-19

Anderson AC, Nicholson LB, Legge KL, Turchin V, Zaghouani H, Kuchroo VK (2000) High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. J. Exp. Med. 191:761-70

Anderton S, Burkhart C, Metzler B, Wraith D (1999) Mechanisms of central and peripheral T-cell tolerance: lessons from experimental models of multiple sclerosis. Immunol. Rev. 169:123-37

Andre I, Gonzalez A, Wang B, Katz J, Benoist C, Mathis D (1996) Checkpoints in the progression of autoimmune disease: lessons from diabetes models. Proc. Natl. Acad. Sci. U. S. A 93:2260-3

Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A (2001) CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J. Immunol. 166:3008-18

Asano M, Toda M, Sakaguchi N, Sakaguchi S (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J. Exp. Med. 184:387-96

Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. Nature 317:359-61

Baker JR, Jr., Saunders NB, Wartofsky L, Tseng YC, Burman KD (1988) Seronegative Hashimoto thyroiditis with thyroid autoantibody production localized to the thyroid. Ann. Intern. Med. 108:26-30

Balasa B, Carayanniotis G (1993) Immunotargeting of thyroglobulin on antigen presenting cells abrogates natural tolerance in the absence of adjuvant. Cell. Immunol. 150:453-8

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. Annu. Rev. Immunol. 18:767-811

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245-52

Banz A, Peixoto A, Pontoux C, Cordier C, Rocha B, Papiernik M (2003) A unique subpopulation of CD4+ regulatory T cells controls wasting disease, IL-10 secretion and T cell homeostasis. Eur. J. Immunol. 33:2419-28

Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, Waal-Malefyt R, Coffman RL, Hawrylowicz CM, O'Garra A (2002) In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J. Exp. Med. 195:603-16

Beisel KW, David CS, Giraldo AA, Kong YM, Rose NR (1982a) Regulation of experimental autoimmune thyroiditis : mapping of susceptibility to the I-A subregion of the mouse H-2. Immunogenetics 15:427-30

Beisel KW, Kong YM, Babu KS, David CS, Rose NR (1982b) Regulation of experimental autoimmune thyroiditis :influence of non-H-2 genes. J. Immunogenetics 9:257-65

Bell D, Young JW, Banchereau J (1999) Dendritic cells. Adv. Immunol. 72:255-324

Bensinger SJ, Bandeira A, Jordan MS, Caton AJ, Laufer TM (2001) Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. J. Exp. Med. 194:427-38

Bernard NF, Ertug F, Margolese H (1992) High incidence of thyroiditis and anti-thyroid autoantibodies in NOD mice. Diabetes 41:40-6

Bluestone JA, Abbas AK (2003) Natural versus adaptive regulatory T cells. Nat. Rev. Immunol. 3:253-7

Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J. Exp. Med. 196:1627-38

Brabb T, Goldrath AW, von Dassow P, Paez A, Liggitt HD, Goverman J (1997) Triggers of autoimmune disease in a murine TCR-transgenic model for multiple sclerosis. J. Immunol. 159:497-507

Braley-Mullen H, Johnson M, Sharp GC, Kyriakos M (1985) Induction of experimental autoimmune thyroiditis in mice with in vitro activated splenic T cells. Cell. Immunol. 93:132-43

Braley-Mullen H, Sharp GC, Bickel JT, Kyriakos M (1991) Induction of severe granulomatous experimental autoimmune thyroiditis in mice by effector cells

activated in the presence of anti-interleukin 2 receptor antibody. J. Exp. Med. 173:899-912

Braley-Mullen H, Sharp GC, Medling B, Tang H (1999) Spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. J Autoimmun 12:157-65

Brand DD, Myers LK, Terato K, Whittington KB, Stuart JM, Kang AH, Rosloniec EF (1994) Characterization of the T cell determinants in the induction of autoimmune arthritis by bovine alpha 1(II)-CB11 in H-2q mice. J. Immunol. 152:3088-97

Braun MC, He J, Wu CY, Kelsall BL (1999) Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor beta1 and beta2 chain expression. J. Exp. Med. 189:541-52

Brocas H, Szpirer J, Lebo RV, Levan G, Szpirer C, Cheung MC, Vassart G (1985) The thyroglobulin gene resides on chromosome 8 in man and on chromosome 7 in the rat. Cytogenet. Cell Genet. 39:150-3

Buelens C, Verhasselt V, De Groote D, Thielemans K, Goldman M, Willems F (1997) Human dendritic cell responses to lipopolysaccharide and CD40 ligation are differentially regulated by interleukin-10. Eur. J. Immunol. 27:1848-52

Buelens C, Willems F, Delvaux A, Pierard G, Delville JP, Velu T, Goldman M (1995) Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. Eur. J. Immunol. 25:2668-72

Canonica GW, Caria M, Torre G, Risso A, Cosulich ME, Bagnasco M (1984) Autoimmune thyroid disease: purification and phenotypic analysis of intrathyroid T cells. J. Endocrinol. Invest 7:641-5

Carayanniotis G (2003) The cryptic self in thyroid autoimmunity: the paradigm of thyroglobulin. Autoimmunity 36:423-8

Carayanniotis G, Chronopoulou E, Rao VP (1994) Distinct genetic pattern of mouse susceptibility to thyroiditis induced by a novel thyroglobulin peptide. Immunogenetics 39:21-8

Carayanniotis G, Kong YC (2000) Pathogenic thyroglobulin peptides as model antigens: insights on the induction and maintenance of autoimmune thyroiditis. Int Rev Immunol 19. 19:557-72

Carayanniotis G, Rao VP (1997) Searching for pathogenic epitopes in thyroglobulin : parameters and caveats. Immunol. Today 18:83-8

Carrasco N (1993) Iodide transport in the thyroid gland. Biochim. Biophys. Acta 1154:65-82

Caturegli P, Vidalain PO, Vali M, Aguilera-Galaviz LA, Rose NR (1997) Cloning and characterization of murine thyroglobulin cDNA. Clin. Immunol. Immunopathol. 85:221-6

Champion BR, Cooke A, Rayner DC (1992) Thyroid autoimmunity. Curr. Opin. Immunol. 4:770-8

Champion BR, Page K, Rayner DC, Quartey-Papafio R, Byfield PG, Henderson G (1987) Recognition of thyroglobulin autoantigenic epitopes by murine T and B cells. Immunology 62:255-63

Champion BR, Page KR, Parish N, Rayner DC, Dawe K, Biswas-Hughes G, Cooke A, Geysen M, Roitt IM (1991) Identification of a thyroxine-containing self epitope of thyroglobulin which triggers thyroid autoreactive T cells. J. Exp. Med. 174:363-70

Chang CH, Furue M, Tamaki K (1995) B7-1 expression of Langerhans cells is up-regulated by proinflammatory cytokines, and is down-regulated by interferon-gamma or by interleukin-10. Eur. J. Immunol. 25:394-8

Charreire J (1989) Immune mechanisms in autoimmune thyroiditis. Adv. Immunol. 46:263-334

Charreire J, Michel-Bechet M (1982) Syngeneic sensitization of mouse lymphocytes on monolayers of thyroid epithelial cells. Eur. J. Immunol. 12:421-5

Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL (1994) Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237-40

Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, Strominger JL (1992) Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature 358:764-8

Christensen HR, Frokiaer H, Pestka JJ (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J. Immunol. 168:171-8

Chronopoulou E, Carayanniotis G (1992) Identification of a thyroiditogenic sequence within the thyroglobulin molecule. J. Immunol. 149:1039-44

Cibotti R, Kanellopoulos JM, Cabaniols JP, Halle-Panenko O, Kosmatopoulos K, Sercarz E, Kourilsky P (1992) Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants. Proc. Natl. Acad. Sci. U. S. A 89:416-20

Consiglio E, Acquaviva AM, Formisano S, Liguoro D, Gallo A, Vittorio T, Santisteban P, De Luca M, Shifrin S, Yeh HJ, . (1987) Characterization of phosphate residues on thyroglobulin. J. Biol. Chem. 262:10304-14

Cottrez F, Hurst SD, Coffman RL, Groux H (2000) T regulatory cells 1 inhibit a Th2-specific response in vivo. J. Immunol. 165:4848-53

Cozzo C, Larkin J, III, Caton AJ (2003) Cutting edge: self-peptides drive the peripheral expansion of CD4+CD25+ regulatory T cells. J. Immunol. 171:5678-82

Croft M (2003) Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat. Rev. Immunol. 3:609-20

Dai Y, Carayanniotis KA, Eliades P, Lymberi P, Shepherd P, Kong Y, Carayanniotis G (1999) Enhancing or suppressive effects of antibodies on processing of a pathogenic T cell epitope in thyroglobulin. J. Immunol. 162:6987-92

Dai YD, Rao VP, Carayanniotis G (2002) Enhanced iodination of thyroglobulin facilitates processing and presentation of a cryptic pathogenic peptide. J. Immunol. 168:5907-11

De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M (1997) Effect of interleukin-10 on dendritic cell maturation and function. Eur. J. Immunol. 27:1229-35

de Vijlder JJ, Den Hartog MT (1998) Anionic iodotyrosine residues are required for iodothyronine synthesis. Eur. J. Endocrinol. 138:227-31

DeGroot LJ (1984) The Thyroid and its diseases. Wiley, New York

DeLisi C, Berzofsky JA (1985) T-cell antigenic sites tend to be amphipathic structures. Proc. Natl. Acad. Sci. U. S. A 82:7048-52

Dhodapkar MV, Steinman RM (2002) Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. Blood 100:174-7

Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J. Exp. Med. 193:233-8

Di Lauro R, Obici S, Condliffe D, Ursini VM, Musti A, Moscatelli C, Avvedimento VE (1985) The sequence of 967 amino acids at the carboxyl-end of rat thyroglobulin. Eur. J. Biochem. 148:7-11

Dunn JT (1995) Thyroglobulin, hormone synthesis and thyroid disease. Eur. J. Endocrinol. 132:603-4

Dunn JT, Anderson PC, Fox JW, Fassler CA, Dunn AD, Hite LA, Moore RC (1987) The sites of thyroid hormone formation in rabbit thyroglobulin. J. Biol. Chem. 262:16948-52

Dunn JT, Kim PS, Dunn AD, Heppner DG, Moore RC (1983) The role of iodination in the formation of hormone-rich peptides from thyroglobulin. J Biol Chem 258:9093-9

Dyson PJ, Knight AM, Fairchild S, Simpson E, Tomonari K (1991) Genes encoding ligands for deletion of V beta 11 T cells cosegregate with mammary tumour virus genomes. Nature 349:531-2

Elrehewy M, Kong YM, Giraldo AA, Rose NR (1981) Syngeneic thyroglobulin is immunogenic in good responder mice. Eur. J. Immunol. 11:146-51

Enk AH, Angeloni VL, Udey MC, Katz SI (1993) Inhibition of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. J. Immunol. 151:2390-8

Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG (1994) Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. Immunogenetics 39:230-42

Faria AM, Weiner HL (1999) Oral tolerance: mechanisms and therapeutic applications. Adv. Immunol. 73:153-264

Farid NR, Sampson L, Moens H, Barnard JM (1981) The association of goitrous autoimmune thyroiditis with HLA-DR5. Tissue antigens 17:265-8

Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, Salomon BL (2003) Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. J. Exp. Med. 198:737-46

Flynn JC, Wan Q, Panos JC, McCormick DJ, Giraldo AA, David CS, Kong YC (2002) Coexpression of susceptible and resistant HLA class II transgenes in murine experimental autoimmune thyroiditis: DQ8 molecules downregulate DR3-mediated thyroiditis. J Autoimmun 18:213-20

Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. 4:330-6

Frankel WN, Rudy C, Coffin JM, Huber BT (1991) Linkage of Mls genes to endogenous mammary tumour viruses of inbred mice. Nature 349:526-8

Fremont DH, Monnaie D, Nelson CA, Hendrickson WA, Unanue ER (1998) Crystal structure of I-Ak in complex with a dominant epitope of lysozyme. Immunity. 8:305-17

Gangi E, Vasu C, Cheatem D, Prabhakar BS (2005) IL-10-producing CD4+CD25+ regulatory T cells play a critical role in granulocyte-macrophage colony-stimulating factor-induced suppression of experimental autoimmune thyroiditis. J. Immunol. 174:7006-13

Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A (2002) Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. Nat. Immunol. 3:33-41

Gillis S, Smith KA (1977) Long term culture of tumour-specific cytotoxic T cells. Nature 268:154-6

Goverman J (1999) Tolerance and autoimmunity in TCR transgenic mice specific for myelin basic protein. Immunol. Rev. 169:147-59

Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM (1993) Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell 72:551-60

Green EA, Gorelik L, McGregor CM, Tran EH, Flavell RA (2003) CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. Proc. Natl. Acad. Sci. U. S. A 100:10878-83

Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737-42

Hajdu A, Rona G (1969) Spontaneous thyroiditis in laboratory rats. Experientia 25:1325-7

Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ (2001) IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J. Immunol. 166:3789-96

Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J. Exp. Med. 194:769-79

Heath VL, Moore NC, Parnell SM, Mason DW (1998) Intrathymic expression of genes involved in organ specific autoimmune disease. J. Autoimmun. 11:309-18

Herzog V (1986) Secretion of sulfated thyroglobulin. Eur. J. Cell Biol. 39:399-409

Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057-61

Hoshioka A, Kohno Y, Katsuki T, Shimojo N, Maruyama N, Inagaki Y, Yokochi T, Tarutani O, Hosoya T, Niimi H (1993) A common T-cell epitope between human thyroglobulin and human thyroid peroxidase is related to murine experimental autoimmune thyroiditis. Immunol. Lett. 37:235-9

Huang FP, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, MacPherson GG (2000) A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. J. Exp. Med. 191:435-44

Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH (1992a) Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science 255:1261-3

Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox AL, Sakaguchi K, Appella E, Grey HM, Sette A (1992b) Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. Science 256:1817-20

Hutchings PR, Cooke A, Dawe K, Champion BR, Geysen M, Valerio R, Roitt IM (1992) A thyroxine-containing peptide can induce murine experimental autoimmune thyroiditis. J. Exp. Med. 175:869-72

Inaba K, Turley S, Yamaide F, Iyoda T, Mahnke K, Inaba M, Pack M, Subklewe M, Sauter B, Sheff D, Albert M, Bhardwaj N, Mellman I, Steinman RM (1998) Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. J. Exp. Med. 188:2163-73

Inaba K, Witmer-Pack M, Inaba M, Hathcock KS, Sakuta H, Azuma M, Yagita H, Okumura K, Linsley PS, Ikehara S, . (1994) The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. J. Exp. Med. 180:1849-60

Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S (1999) Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J. Immunol. 162:5317-26

Itoh Y, Kajino K, Ogasawara K, Katoh M, Namba K, Takami K, Iwabuchi K, Braunstein NS, Onoe K (1996) Determination of the allele-specific antigen-binding site on I-Ak and I-Ab molecules. Eur. J. Immunol. 26:1314-21

Jaeckel E, von Boehmer H, Manns MP (2005) Antigen-specific FoxP3-transduced T-cells can control established type 1 diabetes. Diabetes 54:306-10

Jeker LT, Hejazi M, Burek CL, Rose NR, Caturegli P (1999) Mouse thyroid primary culture. Biochem. Biophys. Res. Commun. 257:511-5

Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC (1995) The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 375:151-5

Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH (2000) Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by

repetitive stimulation with allogeneic immature human dendritic cells. J. Exp. Med. 192:1213-22

Jonuleit H, Schmitt E, Steinbrink K, Enk AH (2001) Dendritic cells as a tool to induce anergic and regulatory T cells. Trends Immunol. 22:394-400

Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, Naji A, Caton AJ (2001) Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat. Immunol. 2:301-6

Kalbus M, Fleckenstein BT, Offenhausser M, Bluggel M, Melms A, Meyer HE, Rammensee HG, Martin R, Jung G, Sommer N (2001) Ligand motif of the autoimmune disease-associated mouse MHC class II molecule H2-A(s). Eur. J. Immunol. 31:551-62

Kalderon AE, Bogaars HA (1977) Immune complex deposits in Graves' disease and Hashimoto's thyroiditis. Am. J. Med. 63:729-34

Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML (1999) T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol. Today 20:561-7

Kamradt T, Mitchison NA (2001) Tolerance and autoimmunity. N. Engl. J. Med. 344:655-64

Kappler J, White J, Wegmann D, Mustain E, Marrack P (1982) Antigen presentation by Ia+ B cell hybridomas to H-2-restricted T cell hybridomas. Proc. Natl. Acad. Sci. U. S. A 79:3604-7

Karras E, Carayanniotis G, Lymberi P (2003) Induction of murine thyroiditis by a non dominant E(k)-restricted peptide of human thyroglobulin. Immunology 108:556-61

Katz JD, Wang B, Haskins K, Benoist C, Mathis D (1993) Following a diabetogenic T cell from genesis through pathogenesis. Cell 74:1089-100

Kawahata K, Misaki Y, Yamauchi M, Tsunekawa S, Setoguchi K, Miyazaki J, Yamamoto K (2002) Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. J. Immunol. 168:4399-405

Khattri R, Cox T, Yasayko SA, Ramsdell F (2003) An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat. Immunol. 4:337-42

Kieffer JD, Mirel RD, Mover H, Erban J, Maloof F (1978) Decreased thyroid function and high plasma prolactin levels in rats of the Buffalo strain. Endocrinology 102:1506-10

Kim PS, Hossain SA, Park YN, Lee I, Yoo SE, Arvan P (1998) A single amino acid change in the acetylcholinesterase-like domain of thyroglobulin causes congenital goiter with hypothyroidism in the cog/cog mouse: a model of human endoplasmic reticulum storage diseases. Proc. Natl. Acad Sci. U. S. A. 95:9909-13

Kim SK, Tarbell KV, Sanna M, Vadeboncoeur M, Warganich T, Lee M, Davis M, McDevitt HO (2004) Prevention of type I diabetes transfer by glutamic acid decarboxylase 65 peptide 206-220-specific T cells. Proc. Natl. Acad. Sci. U. S. A 101:14204-9

Kimura S, Kotani T, McBride OW, Umeki K, Hirai K, Nakayama T, Ohtaki S (1987) Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping, and identification of two alternately spliced mRNAs. Proc. Natl. Acad. Sci. U. S. A 84:5555-9

Kitani A, Chua K, Nakamura K, Strober W (2000) Activated self-MHC-reactive T cells have the cytokine phenotype of Th3/T regulatory cell 1 T cells. J. Immunol. 165:691-702

Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W (2003) Transforming growth factor (TGF)-beta1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. J. Exp. Med. 198:1179-88

Knight SC, Farrant J, Chan J, Bryant A, Bedford PA, Bateman C (1988) Induction of autoimmunity with dendritic cells : studies on thyroiditis in mice. Clin. Immunol. Immunopathol. 48:277-89

Koch F, Stanzl U, Jennewein P, Janke K, Heufler C, Kampgen E, Romani N, Schuler G (1996) High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. J. Exp. Med. 184:741-6

Kohno T, Tsunetoshi Y, Ishikawa E (1988) Existence of anti-thyroglobulin IgG in healthy subjects. Biochem. Biophys. Res. Commun. 155:224-9

Kong YC, McCormick DJ, Wan Q, Motte RW, Fuller BE, Giraldo AA, David CS (1995) Primary hormonogenic sites as conserved autoepitopes on thyroglobulin in murine autoimmune thyroiditis. Secondary role of iodination. J. Immunol. 155:5847-54

Kong YM, David CS, Giraldo AA, Elrehewy M, Rose NR (1979) Regulation of autoimmune response to mouse thyroglobulin : influence of H-2D-end genes. J. Immunol. 123:15-8

Kong YM, David CS, Lomo LC, Fuller BE, Motte RW, Giraldo AA (1997) Role of mouse and human class II transgenes in susceptibility to and protection against mouse autoimmune thyroiditis. Immunogenetics 46:312-7

Kong YM, Lomo LC, Motte RW, Giraldo AA, Baisch J, Strauss G, Hämmerling GJ, David CS (1996) HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice : definitive association with HLA-DRB1*0301 (DR3) gene. J. Exp. Med. 184:1167-72

Kotani T, Umeki K, Yamamoto I, Takeuchi M, Takechi S, Nakayama T, Ohtaki S (1993) Nucleotide sequence of the cDNA encoding mouse thyroid peroxidase. Gene 123:289-90

Kouskoff V, Signorelli K, Benoist C, Mathis D (1995) Cassette vectors directing expression of T cell receptor genes in transgenic mice. J. Immunol. Methods 180:273-80

Kumanogoh A, Wang X, Lee I, Watanabe C, Kamanaka M, Shi W, Yoshida K, Sato T, Habu S, Itoh M, Sakaguchi N, Sakaguchi S, Kikutani H (2001) Increased T cell autoreactivity in the absence of CD40-CD40 ligand interactions: a role of CD40 in regulatory T cell development. J. Immunol. 166:353-60

Kuppers RC, Epstein LD, Outschoorn IM, Rose NR (1994) The IgG2a antibody response to thyroglobulin is linked to the *Igh* locus in mouse. Immunogenetics 39:404-11

Lafaille JJ, Nagashima K, Katsuki M, Tonegawa S (1994) High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. Cell 78:399-408

Lamas L, Anderson PC, Fox JW, Dunn JT (1989) Consensus sequences for early iodination and hormonogenesis in human thyroglobulin. J. Biol. Chem. 264:13541-5

Lardon F, Snoeck HW, Berneman ZN, Van Tendeloo VF, Nijs G, Lenjou M, Henckaerts E, Boeckxtaens CJ, Vandenabeele P, Kestens LL, Van Bockstaele DR, Vanham GL (1997) Generation of dendritic cells from bone marrow progenitors using GM-CSF, TNF-alpha, and additional cytokines: antagonistic effects of IL-4 and IFN-gamma and selective involvement of TNF-alpha receptor-1. Immunology 91:553-9

Lehmann PV, Forsthuber T, Miller A, Sercarz EE (1992) Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 358:155-7

Lehmann PV, Sercarz EE, Forsthuber T, Dayan CM, Gammon G (1993) Determinant spreading and the dynamics of the autoimmune T-cell repertoire. Immunol. Today 14:203-8

Leighton J, Sette A, Sidney J, Appella E, Ehrhardt C, Fuchs S, Adorini L (1991) Comparison of structural requirements for interaction of the same peptide with I-Ek and I-Ed molecules in the activation of MHC class II-restricted T cells. J. Immunol. 147:198-204

Levings MK, Roncarolo MG (2000) T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. J. Allergy Clin. Immunol. 106:S109-S112

Lewis M, Fuller BE, Giraldo AA, Kong YM (1992) Resistance to experimental autoimmune thyroiditis is correlated with the duration of raised thyroglobulin levels. Clin. Immunol. Immunopathol. 64:197-204

Lewis M, Giraldo AA, Kong YM (1987) Resistance to experimental autoimmune thyroiditis induced by physiologic manipulation of thyroglobulin level. Clin. Immunol. Immunopathol. 45:92-104

Lillehoj HS, Beisel K, Rose NR (1981) Genetic factors controlling the susceptibility to experimental autoimmune thyroiditis in inbred rat strains. J. Immunol. 127:654-9

Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM (2002) Immune tolerance after delivery of dying cells to dendritic cells in situ. J. Exp. Med. 196:1091-7

Lohr J, Knoechel B, Jiang S, Sharpe AH, Abbas AK (2003) The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens. Nat. Immunol. 4:664-9

Loser K, Hansen W, Apelt J, Balkow S, Buer J, Beissert S (2005) In vitro-generated regulatory T cells induced by Foxp3-retrovirus infection control murine contact allergy and systemic autoimmunity. Gene Ther. 12:1294-304

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods 223:77-92

Lutz MB, Schuler G (2002) Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. 23:445-9

Lutz MB, Suri RM, Niimi M, Ogilvie AL, Kukutsch NA, Rossner S, Schuler G, Austyn JM (2000) Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. Eur. J. Immunol. 30:1813-22

MacDonald TT (1998) T cell immunity to oral allergens. Curr. Opin. Immunol. 10:620-7

Mahnke K, Knop J, Enk AH (2003a) Induction of tolerogenic DCs: 'you are what you eat'. Trends Immunol. 24:646-51

Mahnke K, Qian Y, Knop J, Enk AH (2003b) Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. Blood 101:4862-9

Mahnke K, Schmitt E, Bonifaz L, Enk AH, Jonuleit H (2002) Immature, but not inactive: the tolerogenic function of immature dendritic cells. Immunol. Cell Biol. 80:477-83

Malthiery Y, Lissitzky S (1987) Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. Eur. J. Biochem. 165:491-8

Many MC, Maniratunga S, Denef JF (1996) The non-obese diabetic (NOD) mouse: An animal model for autoimmune thyroiditis. Exp. Clin. Endocrinol. Diabetes 104 Suppl. 3:17-20

Margalit H, Spouge JL, Cornette JL, Cease KB, DeLisi C, Berzofsky JA (1987) Prediction of immunodominant helper T cell antigenic sites from the primary sequence. J. Immunol. 138:2213-29

Markovic-Plese S, Fukaura H, Zhang J, al Sabbagh A, Southwood S, Sette A, Kuchroo VK, Hafler DA (1995) T cell recognition of immunodominant and cryptic proteolipid protein epitopes in humans. J. Immunol. 155:982-92

Maron R, Cohen IR (1979) Mutation of H-2K locus influences susceptibility to induction of autoimmune thyroiditis. Nature 279:715-6

Maron R, Zerubavel R, Friedman A, Cohen IR (1983) T-lymphocyte line specific for thyroglobulin produces or vaccinates against autoimmune thyroiditis in mice. J. Immunol. 131:2316-22

McHugh RS, Shevach EM (2002) Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. J. Immunol. 168:5979-83

McHugh RS, Shevach EM, Margulies DH, Natarajan K (2001) A T cell receptor transgenic model of severe, spontaneous organ-specific autoimmunity. Eur. J. Immunol. 31:2094-103

McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC (2002) CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity. 16:311-23

McLachlan SM, Rapoport B (1989) Evidence for a potential common T-cell epitope between human thyroid peroxidase and human thyroglobulin with implications for the pathogenesis of autoimmune thyroid disease. Autoimmunity 5:101-6

Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, Erb K, Schuler G, Lutz MB (2002) Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. J. Exp. Med. 195:15-21

Mercken L, Simons M-J, Swillens S, Massaer M, Vassart G (1985) Primary structure of bovine thyroglobulin deduced from the sequence of its 8,431-base complementary DNA. Nature 316:647-51

Misrahi M, Loosfelt H, Atger M, Sar S, Guiochon-Mantel A, Milgrom E (1990) Cloning, sequencing and expression of human TSH receptor. Biochem. Biophys. Res. Commun. 166:394-403

Moens H, Farid NR, Sampson L, Noel EP, Barnard JM (1978) Hashimoto's thyroiditis is associated with HLA-DRw3. N. Engl. J. Med. 299:133-4

Mommaas AM, Mulder AA, Out CJ, Girolomoni G, Koerten HK, Vermeer BJ, Koning F (1995) Distribution of HLA class II molecules in epidermal Langerhans cells in situ. Eur. J. Immunol. 25:520-5

Mori L, Loetscher H, Kakimoto K, Bluethmann H, Steinmetz M (1992) Expression of a transgenic T cell receptor beta chain enhances collagen-induced arthritis. J. Exp. Med. 176:381-8

Morris GP, Chen L, Kong YC (2003) CD137 signaling interferes with activation and function of CD4+CD25+ regulatory T cells in induced tolerance to experimental autoimmune thyroiditis. Cell Immunol. 226:20-9

Mottet C, Uhlig HH, Powrie F (2003) Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J. Immunol. 170:3939-43

Moudgil KD, Sercarz EE (1993) Dominant determinants in hen eggwhite lysozyme correspond to the cryptic determinants within its self-homologue, mouse lysozyme: implications in shaping of the T cell repertoire and autoimmunity. J. Exp. Med. 178:2131-8

Moudgil KD, Sercarz EE (1994) The T cell repertoire against cryptic self determinants and its involvement in autoimmunity and cancer. Clin. Immunol. Immunopathol. 73:283-9

Moudgil KD, Sercarz EE (2005) Understanding crypticity is the key to revealing the pathogenesis of autoimmunity. Trends Immunol. 26:355-9

Mukherjee R, Chaturvedi P, Qin HY, Singh B (2003) CD4+CD25+ regulatory T cells generated in response to insulin B:9-23 peptide prevent adoptive transfer of diabetes by diabetogenic T cells. J. Autoimmun. 21:221-37

Musti AM, Avvedimento EV, Polistina C, Ursini VM, Obici S, Nitsch L, Cocozza S, Di Lauro R (1986) The complete structure of the rat thyroglobulin gene. Proc. Natl. Acad. Sci. U. S. A 83:323-7

Nagayama Y, Kaufman KD, Seto P, Rapoport B (1989) Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. Biochem. Biophys. Res. Commun. 165:1184-90

Nakamura K, Kitani A, Strober W (2001) Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J. Exp. Med. 194:629-44

Nelson CA, Viner NJ, Unanue ER (1996) Appreciating the complexity of MHC class II peptide binding: lysozyme peptide and I-Ak. Immunol. Rev. 151:81-105

Oi VT, Jones PP, Goding JW, Herzenberg LA, Herzenberg LA (1978) Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. Curr. Top. Microbiol. Immunol. 81:115-20

Okayasu I (1985) Transfer of experimental autoimmune thyroiditis to normal syngeneic mice by injection of mouse thyroglobulin-sensitized T lymphocytes after activation with concanavalin A. Clin. Immunol. Immunopathol. 36:101-9

Oldenhove G, de Heusch M, Urbain-Vansanten G, Urbain J, Maliszewski C, Leo O, Moser M (2003) CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. J. Exp. Med. 198:259-66

Olivares-Villagomez D, Wang Y, Lafaille JJ (1998) Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. J. Exp. Med. 188:1883-94

Osman GE, Cheunsuk S, Allen SE, Chi E, Liggitt HD, Hood LE, Ladiges WC (1998) Expression of a type II collagen-specific TCR transgene accelerates the onset of arthritis in mice. Int. Immunol. 10:1613-22

Ozato K, Mayer N, Sachs DH (1980) Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. J. Immunol. 124:533-40

Paget SA, McMaster PR, van Boxel JA (1976) Experimental autoimmune thyroiditis in the guinea pig: characterization of infiltrating lymphocyte populations. J. Immunol. 117:2267-9

Paschke R, Schuppert F, Taton M, Velu T (1994) Intrathyroidal cytokine gene expression profiles in autoimmune thyroiditis. J. Endocrinol. 141:309-15

Penhale WJ, Farmer A, McKenna RP, Irvine WJ (1973) Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. Clin. Exp. Immunol. 15:225-36

Penhale WJ, Irvine WJ, Inglis JR, Farmer A (1976) Thyroiditis in T cell-depleted rats: suppression of the autoallergic response by reconstitution with normal lymphoid cells. Clin. Exp. Immunol. 25:6-16

Penna G, Adorini L (2000) 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J. Immunol. 164:2405-11

Picca CC, Caton AJ (2005) The role of self-peptides in the development of CD4+ CD25+ regulatory T cells. Curr. Opin. Immunol. 17:131-6

Pudifin DJ, Duursma J, Brain P (1977) Experimental autoimmune thyroiditis in the vervet monkey. Clin. Exp. Immunol. 29:256-60

Rachinsky TL, Camp S, Li Y, Ekstrom TJ, Newton M, Taylor P (1990) Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species. Neuron 5:317-27

Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S (1999) SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50:213-9

Rammensee HG, Friede T, Stevanoviic S (1995) MHC ligands and peptide motifs: first listing. Immunogenetics 41:178-228

Rao VP, Balasa B, Carayanniotis G (1994) Mapping of thyroglobulin epitopes: Presentation of a 9mer pathogenic peptide by different mouse MHC class II isotypes. Immunogenetics 40:352-9

Rao VP, Kajon AE, Spindler KR, Carayanniotis G (1999) Involvement of epitope mimicry in potentiation but not initiation of autoimmune disease. J. Immunol. 162:5888-93

Rao VP, Russell RS, Carayanniotis G (1997) Recruitment of multiple V beta genes in the TCR repertoire against a single pathogenic thyroglobulin epitope. Immunology 91:623-7

Rasooly L, Burek CL, Rose NR (1996) Iodine-induced autoimmune thyroiditis in NOD-H-2^{h4} mice. Clin. Immunol. Immunopathol. 31:287-92

Rayner DC, Champion BR, Cooke A (1993) Thyroglobulin as autoantigen and tolerogen. In: Bach J-F (ed) Marcel Dekker, Inc., New York, pp 359-76

Rea D, van Kooten C, van Meijgaarden KE, Ottenhoff TH, Melief CJ, Offringa R (2000) Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. Blood 95:3162-7

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

Roitt IM, Doniach D, Campbell R.N., Hudson R.V. (1956) Autoantibodies in Hashimoto's disease (lymphadenoid goitre). Lancet II820-1

Roitt IM, Jones HE, Mills GL (1965) The activity of different fractions of homologous thyroid extract in the production of allergic thyroiditis in the rat. Immunology 9:281-6

Roman SH, Greenberg D, Rubinstein P, Wallenstein S, Davies TF (1992) Genetics of autoimmune thyroid disease: lack of evidence for linkage to HLA within families. J. Clin. Endocrinol. Metab 74:496-503

Romball CG, Weigle WO (1987) Transfer of experimental autoimmune thyroiditis with T cell clones. J. Immunol. 138:1092-8

Roncarolo MG, Levings MK (2000) The role of different subsets of T regulatory cells in controlling autoimmunity. Curr. Opin. Immunol. 12:676-83

Rose NR (1975) Differing responses of inbred rat strains in experimental autoimmune thyroiditis. Cell. Immunol. 18:360-4

Rose NR, Twarog FJ, Crowle AJ (1971) Murine thyroiditis: importance of adjuvant and mouse strain for the induction of thyroid lesions. J. Immunol. 106:698-704

Rose NR, Witebsky E (1956) Studies on organ specificity. V. Changes in the thyroid glands of rabbits following active immunization with rabbit thryroid extract. J. Immunol. 76:417-27

Rothbard JB, Taylor WR (1988) A sequence pattern common to T cell epitopes. EMBO J. 7:93-100

Rudensky AY, Preston-Hurlburt P, al Ramadi BK, Rothbard J, Janeway CA, Jr. (1992) Truncation variants of peptides isolated from MHC class II molecules suggest sequence motifs. Nature 359:429-31

Sakaguchi S (2004) Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses. Annu. Rev. Immunol. 22:531-62

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. 155:1151-64

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

Salamero J, Remy JJ, Michel-Bechet M, Charreire J (1987) Experimental autoimmune thyroiditis induced by by a 5-10 kDa tryptic fragment from porcine thyroglobulin. Eur. J. Immunol. 17:843-8

Sallusto F, Cella M, Danieli C, Lanzavecchia A (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med. 182:389-400

Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity. 12:431-40

Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N (2000) Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J. Exp. Med. 191:423-34

Scheinecker C, McHugh R, Shevach EM, Germain RN (2002) Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J. Exp. Med. 196:1079-90

Scott CA, Peterson PA, Teyton L, Wilson IA (1998) Crystal structures of two I-Ad-peptide complexes reveal that high affinity can be achieved without large anchor residues. Immunity. 8:319-29

Seddon B, Mason D (1999) Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. J. Exp. Med. 189:877-82

Sercarz EE, Lehmann PV, Ametani A, Benichou G, Miller A, Moudgil K (1993) Dominance and crypticity of T cell antigenic determinants. Annu. Rev. Immunol. 11:729-66

Shevach EM (2000) Regulatory T cells in autoimmmunity*. Annu. Rev. Immunol. 18:423-49

Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S (2002) Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat. Immunol. 3:135-42

Shulman S (1971) Thyroid antigens and autoimmunity. Adv. Immunol. 14:85-185

Silverman DA, Rose NR (1971) Autoimmunity in methylcholanthrene-induced and spontaneous thyroiditis in Buffalo strain rats. Proc. Soc. Exp. Biol. Med. 138:579-84

Simon LL, Justen JM, Giraldo AA, Krco CJ, Kong YM (1986) Activation of cytotoxic T cells and effector cells in experimental autoimmune thyroiditis by shared determinants of mouse and human thyroglobulins. Clin. Immunol. Immunopathol. 39:345-56

Spiro RG, Bhoyroo VD (1988) Occurrence of sulfate in the asparagine-linked complex carbohydrate units of thyroglobulin. Identification and localization of galactose 3-sulfate and N-acetylglucosamine 6-sulfate residues in the human and calf proteins. J. Biol. Chem. 263:14351-8

Steinbrink K, Jonuleit H, Muller G, Schuler G, Knop J, Enk AH (1999) Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood 93:1634-42

Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH (1997) Induction of tolerance by IL-10-treated dendritic cells. J. Immunol. 159:4772-80

Steinman RM, Hawiger D, Nussenzweig MC (2003) Tolerogenic dendritic cells. Annu. Rev. Immunol. 21:685-711

Steinman RM, Nussenzweig MC (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proc. Natl. Acad. Sci. U. S. A 99:351-8

Sternthal E, Like AA, Sarantis K, Braverman LE (1981) Lymphocytic thyroiditis and diabetes in the BB/W rat. A new model of autoimmune endocrinopathy. Diabetes 30:1058-61

Stull SJ, Sharp GC, Kyriakos M, Bickel JT, Braley-Mullen H (1992) Induction of granulomatous experimental autoimmune thyroiditis in mice with in vitro activated effector T cells and anti-IFN-γ antibody. J. Immunol. 149:2219-26

Suri-Payer E, Amar AZ, Thornton AM, Shevach EM (1998) CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. J. Immunol. 160:1212-8

Swiggard WJ, Mirza A, Nussenzweig MC, Steinman RM (1995) DEC-205, a 205-kDa protein abundant on mouse dendritic cells and thymic epithelium that is detected by the monoclonal antibody NLDC-145: purification, characterization, and N-terminal amino acid sequence. Cell Immunol. 165:302-11

Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int. Immunol. 10:1969-80

Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S (2000) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J. Exp. Med. 192:303-10

Tarbell KV, Lee M, Ranheim E, Chao CC, Sanna M, Kim SK, Dickie P, Teyton L, Davis M, McDevitt H (2002) CD4(+) T cells from glutamic acid decarboxylase (GAD)65-specific T cell receptor transgenic mice are not diabetogenic and can delay diabetes transfer. J. Exp. Med. 196:481-92

Taylor BA, Rowe L (1987) The congenital goiter mutation is linked to the thyroglobulin gene in the mouse. Proc. Natl. Acad. Sci. U. S. A 84:1986-90

Texier B, Bédin C, Tang H, Camoin L, Laurent-Winter C, Charreire J (1992) Characterization and sequencing of a 40-amino-acid peptide from human thyroglobulin inducing experimental autoimmune thyroiditis. J. Immunol. 148:3405-11

Thornton AM, Shevach EM (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J. Exp. Med. 188:287-96

Thrasyvoulides A, Sakarellos-Daitsiotis M, Philippou G, Souvatzoglou A, Sakarellos C, Lymberi P (2001) B-cell autoepitopes on the acetylcholinesterase-homologous region of human thyroglobulin: association with Graves' disease and thyroid eye disease. Eur. J. Endocrinol. 145:119-27

Tomazic V, Rose NR (1976) Autoimmune murine thyroiditis VIII. Role of different thyroid antigens in the induction of experimental autoimmune thyroiditis. Immunology 30:63-8

Tomazic V, Rose NR, Shreffler DC (1974) Autoimmune murine thyroiditis. IV. Localization of genetic control of the immune response. J. Immunol. 112:965-9

Tomer Y, Greenberg DA, Concepcion E, Ban Y, Davies TF (2002) Thyroglobulin is a thyroid specific gene for the familial autoimmune thyroid diseases. J. Clin. Endocrinol. Metab 87:404-7

Torrigiani G, Doniach D, Roitt IM (1969) Serum thyroglobulin levels in healthy subjects and in patients with thyroid disease. J. Clin. Endocrinol. Metab. 29:305-14

Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, Cohen JL (2003) Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. J. Clin. Invest 112:1688-96

Tunbridge WM, Evered DC, Hall R, Appleton D, Brewis M, Clark F, Evans JG, Young E, Bird T, Smith PA (1977) The spectrum of thyroid disease in a community: the Whickham survey. Clin. Endocrinol. (Oxf) 7:481-93

Turley SJ, Inaba K, Garrett WS, Ebersold M, Unternaehrer J, Steinman RM, Mellman I (2000) Transport of peptide-MHC class II complexes in developing dendritic cells. Science 288:522-7

Vacchio MS, Berzofsky JA, Krzych U, Smith JA, Hodes RJ, Finnegan A (1989) Sequences outside a minimal immunodominant site exert negative effects on recognition by staphylococcal nuclease-specific T cell clones. J. Immunol. 143:2814-9

van de Graaf SA, Pauws E, de Vijlder JJ, Ris-Stalpers CR (1997) The revised 8307 base pair coding sequence of human thyroglobulin transiently expressed in eukaryotic cells. Eur. J. Endocrinol. 136:508-15

van de Graaf SA, Ris-Stalpers C, Pauws E, Mendive FM, Targovnik HM, de Vijlder JJ (2001) Up to date with human thyroglobulin. J Endocrinol 170:307-21

Van de Keere F., Tonegawa S (1998) CD4(+) T cells prevent spontaneous experimental autoimmune encephalomyelitis in anti-myelin basic protein T cell receptor transgenic mice. J. Exp. Med. 188:1875-82

Van de KF, Tonegawa S (1998) CD4(+) T cells prevent spontaneous experimental autoimmune encephalomyelitis in anti-myelin basic protein T cell receptor transgenic mice. J. Exp. Med. 188:1875-82

Van Heuverswyn B, Leriche A, Van Sande J, Dumont JE, Vassart G (1985) Transcriptional control of thyroglobulin gene expression by cyclic AMP. FEBS Lett. 188:192-6

Van Ommen GJ, Sterk A, Mercken LO, Arnberg AC, Baas F, de Vijlder JJ (1989) Studies on the structures of the normal and abnormal goat thyroglobulin genes. Biochimie 71:211-21

Vanderpump MP, Tunbridge WM, French JM, Appleton D, Bates D, Clark F, Grimley EJ, Hasan DM, Rodgers H, Tunbridge F, . (1995) The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham Survey. Clin. Endocrinol. (Oxf) 43:55-68

Vassart G, Dumont JE (1992) The thyrotropin receptor and the regulation of thyrocyte function and growth. Endocr. Rev. 13:596-611

Vasu C, Dogan RN, Holterman MJ, Prabhakar BS (2003) Selective induction of dendritic cells using granulocyte macrophage-colony stimulating factor, but not fms-like tyrosine kinase receptor 3-ligand, activates thyroglobulin-specific CD4+/CD25+ T cells and suppresses experimental autoimmune thyroiditis. J. Immunol. 170:5511-22

Verginis P, Li HS, Carayanniotis G (2005) Tolerogenic semimature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4+CD25+ T cells. J. Immunol. 174:7433-9

Verginis P, Stanford MM, Carayanniotis G (2002) Delineation of five thyroglobulin T cell epitopes with pathogenic potential in experimental autoimmune thyroiditis. J. Immunol. 169:5332-7

Vladutiu AO, Rose NR (1971) Autoimmune murine thyroiditis : relation to histocompatibility (H-2) type. Science 174:1137-9

Vladutiu AO, Rose NR (1975) Cellular basis of the genetic control of immune responsiveness to murine thyroglobulin in mice. Cell. Immunol. 17:106-13

Vladutiu AO, Steinman L (1987a) Inhibition of experimental autoimmune thyroiditis in mice by anti-I-A antibodies. Cell. Immunol. 109:169-80

Vladutiu AO, Steinman L (1987b) Inhibition of experimental autoimmune thyroiditis in mice by anti-I-A antibodies. Cell Immunol. 109:169-80

Volpe R (1991) Autoimmune diseases of the endocrine system. Boca Raton : CRC Press,

von Boehmer H (1990) Developmental biology of T cells in T cell-receptor transgenic mice. Annu. Rev. Immunol. 8:531-56

Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H (2003) Characterization of Dendritic Cells that Induce Tolerance and T Regulatory 1 Cell Differentiation In Vivo. Immunity. 18:605-17

Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK (2003) Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. J. Exp. Med. 198:249-58

Wan Q, Shah R, Panos JC, Giraldo AA, David CS, Kong YM (2002) HLA-DR and HLA-DQ polymorphism in human thyroglobulin-induced autoimmune thyroiditis: DR3 and DQ8 transgenic mice are susceptible. Hum Immunol 63:301-10

Watanabe H, Inaba M, Adachi Y, Sugiura K, Hisha H, Iguchi T, Ito T, Yasumizu R, Inaba K, Yamashita T, Ikehara S (1999) Experimental autoimmune thyroiditis induced by thyroglobulin-pulsed dendritic cells. Autoimmunity 31:273-82

Weetman AP, McGregor AM (1984) Autoimmune thyroid disease: developments in our understanding. Endocr. Rev. 5:309-55

Weetman AP, McGregor AM (1994) Autoimmune thyroid disease: further developments in our understanding. Endocr. Rev. 15:788-830

Weiner HL (2001) Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. Microbes. Infect. 3:947-54

White J, Blackman M, Bill J, Kappler J, Marrack P, Gold DP, Born W (1989) Two better cell lines for making hybridomas expressing specific T cell receptors. J. Immunol. 143:1822-5

Wick G, Beutner EH, Nilsson LA (1971) Evaluation of spontaneous autoimmune thyroiditis in chickens of the obese strain (OS) by defined immunofluorescence. Ann. N. Y. Acad. Sci. 177:175-82

Wick G, Boyd R, Hala K, Thunold S, Kofler H (1982) Pathogenesis of spontaneous autoimmune thyroiditis in Obese strain (OS) chickens. Clin. Exp. Immunol. 47:1-18

Wick G, Sundick RS, Albini B (1974) A review: The obese strain (OS) of chickens: an animal model with spontaneous autoimmune thyroiditis. Clin. Immunol. Immunopathol. 3:272-300

Witebsky E, Rose NR (1956) Studies on organ specificity IV. Production of rabbit thyroid antibodies in the rabbit. J. Immunol. 76:408-16

Yamaguchi Y, Tsumura H, Miwa M, Inaba K (1997) Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. Stem Cells 15:144-53

Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, Steinman RM (2003) Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. J. Exp. Med. 198:235-47

Yewdell JW, Frank E, Gerhard W (1981) Expression of influenza A virus internal antigens on the surface of infected P815 cells. J. Immunol. 126:1814-9

Yu P, Gregg RK, Bell JJ, Ellis JS, Divekar R, Lee HH, Jain R, Waldner H, Hardaway JC, Collins M, Kuchroo VK, Zaghouani H (2005) Specific T regulatory

cells display broad suppressive functions against experimental allergic encephalomyelitis upon activation with cognate antigen. J. Immunol. 174:6772-80

Zal T, Volkmann A, Stockinger B (1994) Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. J. Exp. Med. 180:2089-99

Zamvil SS, Steinman L (1990) The T lymphocyte in experimental allergic encephalomyelitis. Annu. Rev. Immunol. 8:579-621

Zhu Y, Rudensky AY, Corper AL, Teyton L, Wilson IA (2003) Crystal structure of MHC class II I-Ab in complex with a human CLIP peptide: prediction of an I-Ab peptide-binding motif. J. Mol. Biol. 326:1157-74