THE ROLE OF IODINE IN EXPERIMENTAL AUTOIMMUNE THYROIDITIS

HAIYAN S. LI

The role of iodine in experimental autoimmune thyroiditis

BY

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ABSTRACT

Experimental autoimmune thyroiditis (EAT), a murine model of Hashimoto's thyroiditis in humans, is a T cell-mediated autoimmune disease, characterized by destruction of thyroid follicles by infiltrating inflammatory cells. Immunization with thyroglobulin (Tg) - one of the major thyroid autoantigens – or Tg peptides in complete Freund's adjuvant is the classical way to elicit EAT in susceptible mouse strains.

The first part of the thesis investigates the critical role of iodine in the immunopathogenicity of Tg. I have delineated three iodotyrosyl-containing peptides (aa. 117-132, 304-318, and 1931-1945) which are not immunogenic in their native form but become immunopathogenic in their iodinated form. Iodination of tyrosyls facilitates either peptide binding to MHC or T-cell recognition of the peptide. In addition, iodotyrosyl formation has increasing, neutral or decreasing effects on the immunogenic profiles of other three Tg peptides (a.a. 179-194, 2529-2545, and 2540-2554) which are immunogenic in their non-iodinated forms. In a parallel study, I attempt to generate highly iodinated Tg in vivo via NaI administration in the drinking water of mice. We found that this regimen did not facilitate the generation of highly iodinated Tg in vivo, but elicited goitrous hypothyrodism in SJL but not CBA/J mice. The mechanisms behind this phenomenon remain poorly understood, but it does not seem to have an autoimmune basis.

In the second part of the thesis, I examined whether release of tissue antigens from necrotic thyroid epithelial cells can trigger dendritic cell (DC) maturation and initiation of a primary anti-self response. We found that exposure to necrotic – but not viable - thyrocytes ex vivo triggered phenotypic and functional maturation of bone marrow-derived DC. This enabled the immunogenic presentation of thyroid antigens, such as Tg, on the DC surface, leading to the development of EAT. These results support the view that thyroid epithelial cell necrosis may cause autoimmune thyroiditis via maturation of intrathyroidal DC.

Finally, I have examined the intrathymic presence of mRNA transcripts of mouse Tg which encode thirteen pathogenic peptides, scattered over a large (8.5 kb) sequence. We found that Tg mRNA transcripts in thymus, liver and kidney lack the 1-915 bp (including peptide 1-12) and 961-5013 bp (including peptide 1579-91) segments, spanning exons 1-7 and 9-22, respectively. These data demonstrate that certain known and perhaps other as yet unmapped pathogenic T-cell epitopes of Tg cannot be encoded by the truncated isoform(s) of intrathymic Tg mRNA. These findings also imply that central tolerance to endogenous Tg produced by thymic epithelial cells may be incomplete. It is not known, however, to what extent blood-borne Tg molecules that leak from the thyroid in small amounts contribute to the process of tolerance induction.

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Abbreviations

a.a.	Amino acid
A-Ab	Autoantibody
A-Ag	Autoantigen
Ab	Antibody
ADCC	Antibody-dependent cell mediated cytotoxicity
Ag	Antigen
AITD	Autoimmune thyroid disease
APC	Antigen presenting cell
AT	Autoimmune thyroiditis
ATCC	American Type Culture Collection
BM	Bone marrow
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CTLA-4	The Cytotoxic T-lymphocyte Antigen-4
DC	Dendritic cells
DIT	Diiodotyrosine

DMEM	Dulbecco's modified Eagle's medium
DTR	Diphtheria toxin receptor
DUOX	Dual oxidase
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
EAU	Experimental autoimmune uveitis
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAD	Glutamic acid decarboxylase
GD	Graves' disease
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HI-Tg	Highly iodinated Tg
HLA	Human leukocyte antigen
HT	Hashimoto's thyroiditis

IDDM	Insulin-dependent diabetes mellitus
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon-γ
Ig	Immunoglobulin
I.I.	Infiltration index
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
IRBP	Interphotoreceptor retinoid-binding protein
i.p.	Intraperitoneal
i.v.	Intravenous
kDa	Kilodalton
LCMV-GP	Lymphocytic choriomeningitis virus glycoprotein
LI-Tg	Thyroglobulin depleted of iodine
LNC	Lymph node cells
LPS	Lipopolysaccharide

LT	Lymphocytic thyroiditis
mAb	Monoclonal antibody
MBP	Myelin basic protein
2-ME	2-mercaptoethanol
MHC	Major histocompatibility complex
MIT	Monoiodotyrosine
mRNA	Messenger RNA
NADPH	Thyroid nicotinamide adenine dinucleotide phosphate
NaI	Sodium iodide
NIS	Sodium iodide symporter
NI-Tg	Normally iodinated Tg
NOD	Non obese diabetic
NT	Necrotic thyrocytes
NT/DC	Dendritic cells exposed to necrotic thyrocytes
NTx	Neonatal thymectomy
OS	Obese Strain
OVA	Ovalbumin

OVA/DC	Dendritic cell exposed to ovalbumin
PBS	Phosphate buffered saline
PLP	Proteolipid protein
RBC	Red blood cells
SAT	Spontaneous autoimmune thyroiditis
s.c.	Subcutaneous
S.I.	Stimulation index
T0	Thyronine
T3	Triiodothyronine
T4	Thyroxine
TBAb	Thyroid-blocking Ab
TEC	Thyroid epithelial cells
Tg	Thyroglobulin
Tg/DC	Dendritic cell exposed to thyroglobulin
THOX	Thyroid nicotinamide adenine dinucleotide phosphate oxidase
TPO	Thyroid peroxidase
Treg	Regulatory T cells

TsAb	Thyroid-stimulating A-Abs
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone-receptor
TCR	T cell receptor
TGF-β	Transforming growth factor-β
VT	Viable thyrocytes
VT/DC	Dendritic cell exposed to viable thyrocytes

CHAPTER 1

INTRODUCTION & OVERVIEW

1.1 THE ANATOMY AND FUNCTION OF THE THYROID

The thyroid gland is the largest organ that functions exclusively as an endocrine gland in humans. It is located in the neck, below and in front of the larynx, and contains two lobes connected by the isthmus. The gland is composed of follicles with varying sizes that contain a colloid-filled lumen surrounded by a single layer of epithelial cells (follicular cells) enclosed by a basement membrane where parafollicular cells reside (Capen 2000).

The function of the thyroid is to secrete thyroid hormones, triiodothyronine (T3) and thyroxine (T4), which are essential for the regulation of metabolic processes throughout the body. The biosynthesis of thyroid hormones requires the availability of exogenous iodine and protein matrix, the thyroglobulin (Tg). Thyroglobulin is synthesized on the rough endoplasmic reticulum (ER), packaged in the large Golgi apparatus of follicular cells, then secreted and stored as colloid in the lumen. Inorganic iodide in the thyroid comes from two distinct sources: iodide trapped from interfollicular capillaries and actively transported by sodium iodide symporter (NIS) across the basolateral membrane of follicular cells (Spitzweg and Morris 2002), or internal iodide produced by deiodination of organic iodine compounds. Another chloride -iodide transporter, pendrin,

probably mediates the subsequent transportation of iodide from the apical membrane to the follicular lumen (Royaux et al. 2000).

Human Tg contains 66 Tyr residues per monomeric unit and some of them are subject to iodination under physiological conditions. Within the follicular lumen, iodide is rapidly oxidized and binds to tyrosyl residues in Tg, generating monoiodotyrosine (MIT) and diiodotyrosine (DIT). A few specific iodotyrosyl precursors will serve as acceptor and donor to synthesize the thyroid hormones, T3 and T4, through intramolecular coupling (Dunn and Dunn 2000). This iodination process occurs at the apical plasma membrane-follicle lumen boundary and is mediated by thyroid peroxidase (TPO) in the presence of an H_2O_2-generating system. In the thyroid, the H_2O_2 generator is the thyroid nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (THOX) (De Deken et al. 2000, Dupuy et al. 2000), designated as Dual Oxidase (DUOX) by The HUGO Human Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/ nomenclature).

Hormone-containing Tg molecules are resorted from the colloid and transported from the apical to the basolateral surface of the follicular cells. Enzymatic degradation by intracellular lysosomes frees the T4 and T3 molecules contained within Tg, with T4 being the preferred secretory product. T4 and T3 are eventually secreted into the extracellular space. Synthesis of thyroid hormones is stimulated by the pituitary-derived thyroid stimulating hormone (TSH), which interacts with the TSH receptor (TSHR) at the basolateral membrane of the thyrocytes (Carrasco et al. 2000).

T4 is a pro-hormone, with little intrinsic hormonal activity, and it must be converted to the potent T3 to render biological action. These thyroid hormones have important effects on development, growth, and metabolism. In childhood, lack of thyroid hormone can cause delayed growth. The effects are probably metabolic rather than developmental, as growth is restored rapidly after the institution of thyroid hormone treatment. In adults, the primary effects of thyroid hormones are manifested by alterations in metabolism, such as changes in oxygen consumption, protein, carbohydrate, lipid, and vitamin metabolism (Capen 2000).

1.2 AUTOIMMUNE THYROIDITIS (AT)

1.2.1 Autoimmune thyroid disease (AITD)

Autoimmune disease occurs when the body's immune system becomes misdirected, attacking self-tissue Ags. Autoimmune diseases involve either a specific organ (organ-specific autoimmune disease) or many tissues of the body (systemic autoimmune disease), and are often demonstrated by the presence of autoantibodies (A-Abs) or self-reactive T lymphocytes. Autoimmune thyroid disease (AITD) is a thyroid-specific autoimmune disorder, affecting women 5-10 times as often as men. AITDs encompass a diverse range of clinical entities, including Grave's disease (GD, a form of hyperthyroidism or excessive thyroid hormone production), Hashimoto's thyroiditis (HT, a form of hypothyroidism with deficient thyroid hormone production), and primary thyroid failure or myxedema. All these disorders share the common histological features of thyroid lymphocytic infiltration, but result in diametrically opposed endocrine outcomes.

HT is also named autoimmune thyroiditis (AT) or lymphocytic thyroiditis (LT). The major characteristic of HT is the inflammatory infiltration of mononuclear cells that replace the parenchyma, which eventually leads to gland fibrosis. Progressive loss of thyroid cells and gradual destruction of the gland lead to thyroid hormone deficiency and clinical hypothyroidism. Subclinical HT is determined by an elevated serum TSH concentration (>4.8 mIU/L) but with a normal range of free T3 and T4. Under clinical presenting (overt) hypothyroidism, serum concentrations of both TSH and thyroid

hormones are out of the normal range (Hueston 2001). The immunopathology of HT is highlighted with mononuclear cell infiltration of the thyroid gland and the production of A-Abs. Diagnosis of HT is usually made by the finding of A-Abs to TPO or Tg in serum, but the presence of such A-Abs does not necessarily indicate the occurrence of HT as they are present in 5-10% of normal individuals without thyroid damage (Weetman and McGregor 1994).

1.2.2 Experimental autoimmune thyroiditis

Experimental autoimmune thyroiditis (EAT), the animal model of HT, can be experimentally induced or spontaneously develop in susceptible animals. These models provide valuable tools to investigate the pathogenesis and immunoregulation of AITD. However, animals with EAT may not develop hypothyroidism. The criteria for EAT diagnosis include: (1) circulating anti-Tg Abs and, (2) lymphocytic infiltration of the thyroid gland. Of note, these two criteria for EAT are not equivalent; EAT can develop in the absence of A-Ab production (Salamero et al. 1987, Verginis et al. 2002). EAT closely resembles the autoimmune characteristics of HT but differs in a few aspects as discussed below.

Direct EAT induced by immunization with thyroid autoantigens

In 1956, EAT was first described in a pioneering study performed by Witebsky and Rose (Rose and Witebsky 1956, Witebsky and Rose 1956). In their studies, EAT - as revealed

by mononuclear infiltration of the thyroid gland and A-Ab production - was induced in rabbits by subcutaneous injection of homologous thyroid extracts together with complete Freund adjuvant (CFA), which contains inactivated mycobacteria in mineral oil. EAT was later induced in a variety of animal species such as dog (Terplan et al. 1960), guinea pig (Terplan et al. 1960), rat (Jones and Roitt 1961), monkey (Kite et al. 1966) and mouse (Rose et al. 1971) by injection of homologous or heterologous thyroid extract in adjuvant. Moreover, Shulman *et al.* have shown that EAT can be induced by the soluble fraction of the thyroid gland, where Tg represents 75-80% of the protein (Shulman 1971). Other early studies used heterologous or chemically modified Tg to induce thyroiditis in rabbits (Weigle 1965, Kite et al. 1966, Mangkornkanok et al. 1972). When homologous and heterologous Tg preparations were used simultaneously for EAT induction (Romball and Weigle 1984), it was found heterologous Tg is more immunogenic but less thyroiditogenic than self Tg. It was proposed later by Wan *et al.* that certain epitopes within the Tg conserved between species may be more thyroiditogenic than unique species-specific epitopes (Wan et al. 1997).

In the above studies, a strong adjuvant, such as CFA or lipopolysaccharide (LPS), is required for induction of thyroid autoantigen (A-Ag)-specific immune responses. Interestingly, repeated intravenous injections of foreign (Terplan et al. 1960) or self Tg (Eirehewy et al. 1981), or altered Tg coupled to diazonium derivatives of arsenilic and sulfanilic acid (Weigle 1965), into rabbits or good responder mice sometimes elicited thyroid autoimmunity in the absence of adjuvant. Furthermore, injection of Tg conjugated to anti-A^k monoclonal Abs (mAbs) without adjuvant by Balasa *et al.* successfully induced

host IgG-responses, but failed to induce mononuclear infiltration of the thyroid, thereby suggesting lack of activation of T-cell subsets with pathogenic potential in EAT (Balasa and Carayanniotis 1993).

Among these various methods, a commonly used procedure was subcutaneous injection of self Tg mixed in CFA followed by a booster injection with Tg in incomplete Freund adjuvant (IFA) in 2-3 week intervals (Weetman and McGregor 1984, Weetman and McGregor 1994, Charreire 1989). Lastly, Tg peptides, derived from either human or mouse Tg sequences, can also cause EAT in susceptible animals either by injection of peptide in CFA or by adoptive transfer of peptide-primed lymph node cells (LNC) after in vitro pulsing with the priming peptide (Carayanniotis 2003). Of note, none of these pathogenic Tg peptides has been classified as immunodominant since they were not readily processed and presented from intact Tg in vivo and in vitro as revealed by LNC recall assays (Carayanniotis 2003).

Other thyroid A-Ags have also been used for EAT induction. For example, immunization with crude thyroid microsomal preparations was shown to induce thyroiditis in rabbits or Rhesus monkeys (Weigle 1965, Kite et al. 1966, Mangkornkanok et al. 1972). It has been clarified that TPO is the primary thyroid microsomal Ag involved in AITD (Czarnocka et al. 1985, Kohno et al. 1986, Kotani et al. 1986). The development of EAT induced with TPO has been difficult, principally due to difficulties in purifying substantial quantities of TPO and excluding Tg contamination. An alternative source is recombinant human TPO

(rhTPO) prepared in eukaryotic expression systems such as insect cells, but rhTPO is poorly glycosylated and not fully enzymatically active with significant contamination of denatured TPO (Gardas et al. 1999). Despite these difficulties, murine EAT was successfully elicited in C57BL/6 mice (H-2^b) by challenging them with either porcine TPO purified from thyroid by trypsinization (Kotani et al. 1990), or a 15 mer TPO peptide (Kotani et al. 1992). In a recent study, a variety of TPO preparations - purified hTPO, insect-derived rhTPO, trypsinized porcine TPO and plasmid-encoded hTPO - were used to thoroughly investigate the thyroiditogenic role of these TPO preparations in DR3-transgenic mice (Flynn et al. 2004a). The results showed that plasmid DNA encoding hTPO was superior to other TPO preparations in eliciting thyroid autoimmunity, in terms of both anti-TPO Ab responses and infiltration of the gland.

Besides Tg and TPO, TSHR is a third A-Ag within the thyroid. In 1994, a new animal model was established showing the development of AITD in Balb/c mice immunized with the extracellular domain of human TSHR expressed as a fusion protein linked to a maltose-binding protein (Costagliola et al. 1994). Fifty percent of immunized Balb/c mice (H-2^d) had thyrotropin-binding inhibiting A-Abs, accompanied by low T4 levels and lymphocytic infiltration in the thyroid. Later studies found that NOD mice immunized with TSHR display severe thyroid infiltration of immune cells, predominantly T cells, but there was lack of A-Ab production (Many et al. 1996).

Of particular note, signs of hypothyroidism were rarely evaluated in animals with induced EAT, and it is unclear whether thyroid functions are altered in most of these animals. However, one study reported T4 levels and iodine uptake were significantly decreased in susceptible mice from 2 to 8 weeks after immunization with Tg in CFA (Vladutiu and Kenney 1985). In rats, serum Tg and TSH levels were strongly augmented to approximately 10 times above the basal level at 5 weeks postimmunization with Tg (Hassman et al. 1988).

Indirect EAT induced by transfer or depletion of lymphocyte subsets

Penhale *et al.* have shown that typical EAT can be induced in rats depleted of T lymphocytes by neonatal thymectomy (NTx), followed by several low doses of irradiation (Penhale et al. 1973, Penhale et al. 1976). This disease can be ameliorated by reconstitution with syngeneic lymphocytes. Similarly, Wick et al (Wick et al. 1974) reported that Tx of newly hatched Obese Strain (OS) chicken – which develop spontaneous thyroiditis - accelerated and aggravated the lymphoid infiltration of the thyroid gland. These experiments provided basic support for the argument that EAT can be caused by selective depletion of a T cell subpopulation responsible for the suppression of thyroid-specific autoreactivity, which is now known as T regulatory cells (Treg).

The second approach, using T cells as EAT inducers, was demonstrated using adoptive transfer experiments. In numerous experiments, EAT was induced in mice or guinea pigs using syngeneic spleen or LNC previously stimulated by Tg (Braley-Mullen et al. 1981,

Okayasu 1985, Simon et al. 1986) or syngeneic thyroid epithelial cells (TEC) (Charreire and Michel-Bechet 1982). Similarly, transfer of Tg-specific T-cell clones or lines into naïve recipients induced development of lymphocytic infiltration in the thyroid (Maron et al. 1983, Romball and Weigle 1987), further supporting the effector role of T cells in EAT induction. According to another study, Tg-primed T-cells which were activated in vitro with concanavalin A (con A), were also able to transfer EAT (Okayasu 1985). Moreover, several studies have shown that Tg peptide-primed-LNC after restimulation in vitro with the respective peptide were also able to transfer EAT in syngeneic naïve recipients (Carayanniotis and Rao 1997). A more severe form of EAT, called granulomatous thyroiditis, can be induced in naive recipient mice by adoptive transfer of mTg-primed spleen cells activated in vitro in the presence of either anti-IL-2R (Braley-Mullen et al. 1991), anti-IFN-γ mAbs (Stull et al. 1992) or IL-12 (Braley-Mullen et al. 1998).

Professional antigen presenting cells (APC) loaded with A-Ags have also been used to elicit EAT. Intravenous (i.v.) injection of 10^5 dendritic cells (DC), either pulsed with large amounts of Tg in vitro, or obtained from the spleens of Tg-primed mice, can induce thyroiditis in recipient hosts (Knight et al. 1988). Interestingly, A-Ab to Tg were only detected in mice that received DC loaded with Tg in vivo, but not in vitro. More recently, DC purified from high responder B10BR mice were pulsed in vitro with porcine Tg, then transferred into syngeneic mice, resulting in the development of thyroiditis and IgG2a A-Abs, indicating the activation of Th1 cells (Watanabe et al. 1999).

Comparisons of EAT developed after injection of Tg in CFA to that induced by transfer of Tg-specific T cells or Tg-pulsed DC showed that a uniform feature of the disease is lymphocytic infiltration of the thyroid gland. The presence of the A-Ab is a variable parameter, independent of the severity of the disease. Among the animal models of induced EAT, the mouse model is the most extensively studied due to the extensive characterization of the MHC molecules and the great availability of congenic, intra H-2-recombinant and congenic mutant strains. Also, mice can be easily handled and maintained in large numbers at a lower cost than any other animals developing EAT. Furthermore, there is an enormous variety of mouse-specific reagents such as Abs specific for cell surface markers or cytokines which can be used in phenotypic and functional analyses of the cells involved in the immune response.

Spontaneous animal model of thyroiditis

Spontaneous autoimmune thyroiditis (SAT) has been reported in the OS chicken, non-obese diabetic (NOD) mice, Bio-breeding/Worchester (BB/W) and buffalo (BUF) rats, as well as Praomys, a desert rodent, as reviewed in (Charreire 1989).

The OS chicken

The first established and best studied model of SAT is the OS chicken, which was derived from Cornell-C Strain (CS) chicken by selective breeding for hypothyroidism (Cole 1966). The OS chicken develops SAT during the first 3 weeks after hatching, which

closely resembles the human HT in all clinical, histopathological, endocrinological and serological aspects. SAT in OS chicken is characterized by massive infiltration of the thyroid gland by mononuclear cells and the occurrence of large numbers of germinal center, with consequent hypothyroidism, as well as circulating A-Abs to Tg and TPO. The phenotypic symptoms in these chickens are small body size, abundant subcutaneous and abdominal fat deposits, cold sensitivity and long silky feathers (Wick et al. 1982).

OS chickens have abnormal immune system and thyroid gland functions , which may link to SAT (Sundick et al. 1996, Wick et al. 1982, Wick et al. 1989), including: (1) a general T lymphocyte hyperactivity, accompanied by reduced size, weight and total cell number of lymphoid organs; (2) a significant increase in IgM-positive B cells in lymphoid organs, especially in the thymus; (3) a hyperactive thyroid gland: elevated iodine uptake, elevated release of organified iodine, leading to low iodine content in Tg; (4) a deficiency of the neuroendocrine feedback control of the thyroid gland. These defects were suggested to play a potential etiological role in SAT.

The OS chicken has proven a particularly useful animal model for HT for two reasons. First, germinal centers, a hallmark of HT but absent in induced EAT, develop at 6-8 weeks of age and replace functional thyroid tissue, resulting in fibrosis of the thyroid gland (Rose et al. 1981, Wick et al. 1982). Second, at the time of hatching, immune complexes consisting of Tg and maternal anti-Tg Abs deposit in the basal lamina of

thyroid gland, which could prevent the immune system from interacting with these Ags during the tolerance induction period (Charreire 1989).

The NOD mouse

The NOD is an inbred strain developed from a cataract-prone subline of outbred ICR mice, and has been a useful model for insulin-dependent diabetes mellitus (IDDM). Bernard *et al.* have reported a very high incidence (67-90%) of thyroiditis in 2-25 week old female and male NOD mice, with the presence of circulating A-Abs to TPO in 35% of the mice (Bernard et al. 1992). In addition, the severity of thyroiditis was not significantly different between NOD diabetic and nondiabetic mice. However, the incidence of thyroiditis, as that of diabetes, can vary among colonies, and a very low (< 5%) incidence of thyroiditis has been reported in NOD mice of similar age from two different colonies (Many et al. 1996).

Recently, a NOD MHC congeneic strain, NOD.H-2^{h4}, was derived by crossing NOD to B10.A(4R) mice with repetitive backcrosses to NOD. These mice express H-$2K^{k}$ and I-A^{k} on the NOD background and spontaneously develop thyroid lesions and anti-Tg A-Abs. Although 20-30% of NOD.H-2^{h4} mice develop insulitis, they do not develop diabetes (Weatherall et al. 1992). About 60-70% of these mice develop SAT at 7-10 months of age, and iodine intake enhances A-Ab levels and incidence of thyroiditis to nearly 100% (Braley-Mullen et al. 1999, Rasooly et al. 1996). However, these mice maintain normal

levels of thyroid hormones, even after iodine administration (Yu et al. 2006b). Essentially, all NOD.H-2^{h4} mice that developed thyroid lesions produced readily detectable IgG A-Abs against Tg, primarily of IgG2a and IgG2b subclasses (Rasooly et al. 1996).

BUF and BB/W Rats

In 1969, BUF rats were first reported to exhibit spontaneous histopathological changes of their thyroids comparable to those of HT (Rasooly et al. 1996). Subsequent studies by Silverman and Rose (Silverman and Rose 1971, Silverman and Rose 1975) demonstrated that 14% of female BUF rats developed SAT between 9-12-weeks of age, accompanied by the formation of intrathyroidal germinal centres. The incidence and severity of thyroiditis can be greatly increased in both males and females by neonatal thymectomy or simultaneous administration of Tg (Silverman and Rose 1974a) and other agents (Silverman and Rose 1974b). The NTx-induced EAT in BUF rats is a transient disease (Cohen et al. 1988), which closely resembles human HT in terms of the level of anti-Tg A-Abs, enlargement of the thyroid gland, elevated TSH and severity of the disease (Cohen and Weetman 1987).

Another strain of rat, the BB/W, was found to develop SAT in parallel to IDDM, similarly as the NOD mice (Sternthal et al. 1981). However, the incidence of SAT was strikingly higher in 8-10-month old diabetic rats (59%), as compared to their nondiabetic

cohorts (11%). Thyroiditis in diabetic BB/W rats was not accompanied by any consistent changes in serum T4, T3, and TSH concentrations.

1.2.3 The major thyroid A-Ags: Tg, TPO, TSHR

As mentioned above, Tg, TPO and TSHR are the major thyroid A-Ags identified in AITD. Although autoreactivity has been demonstrated to all three A-Ags in both HT and GD, Abs to the first two Ags are frequently detected in patients with HT and, the last one is believed to be the main Ag in GD. Recently, NIS was proposed as another potential A-Ag in AITD (Spitzweg and Morris 2002, Sternthal et al. 1981).

Tg and its epitope mapping

Tg represents the most abundant Ag of the thyroid gland and provides a matrix for incorporation and storage of available inorganic iodide for subsequent synthesis of the thyroid hormones (Section 1.1.2). It is the largest A-Ag known, consisting of two monomeric polypeptide chains joined together by disulfide bonds, which make up a mature 660 kDa, 19S dimer. Tg is not a sequestered Ag 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).

To date, the complete primary structure of human (Malthiery and Lissitzky 1987), bovine (Mercken et al. 1985), rat (Musti et al. 1986) and mouse (Caturegli et al. 1997, Kim et al.

1998) Tg have been deduced from their respective mRNAs. Partial sequences of Tg from several other species, including goat (van Ommen et al. 1989), sheep (Durand et al. 1987), rabbit (Dunn et al. 1987) and turtle (Roe et al. 1989), have been reported. The Tg gene is localized on different chromosomes among species, for example, it is found on chromosome 8 in humans (Brocas et al. 1985), chromosome 15 in mice (Taylor and Rowe 1987) and chromosome 7 in rats (Brocas et al. 1985). Tg is a highly conserved protein, and identity at the a.a. level among different species is above 70% (Vali et al. 2000). An analysis of the primary a.a. sequence of Tg reveals a highly organized internal structure. The amino portion of the molecule (approximately, a.a. 1-2170) shows a high degree of internal homology with three types of repetitive domains, in which the positions of cysteine and, to a lesser extent, proline and glycine residues are conserved (Malthiery and Lissitzky 1987, Mercken et al. 1985). The repetitive structure suggests that this part of the Tg gene evolved as a result of the serial duplication of 3 basic primordial genes, and was hypothesized to be involved in proteolytic processing (Parma et al. 1987). In contrast, the C-terminal portion (~ 550 a.a.) contains no internal repetitive domains, but shows ~ 28% of homology to acetylcholinesterase (AchE), a type B carboxylesterase (Swillens et al. 1986), which indicates this portion of Tg may have a function similar to that of AchE for cell membrane binding. The absence of homology between the amino and carboxyl portions of Tg suggests they arose from different ancestral genes.

Tg is a highly glycosylated molecule with carbohydrate moieties, making up ~ 10% of its mass (Charreire 1989). The polypeptide chain of Tg is synthesized on the surface of rough ER in thyroid follicular cells. Then it is directed into the ER lumen by a signal

peptide which contains the first 19 or 20 a.a. (Ring et al. 1987). As this translocation occurs, Tg undergoes a series of conformational changes aided by folding enzymes. Upon formation of stable dimers, the nascent proteins migrate to the Golgi complex where addition of carbohydrate units are completed (glycosylation) (Ring et al. 1987), and sulfation occurs (Herzog 1986). Phosphorylation of Tg also appears to be an intracellular event, although the precise sub-cellular compartment has not been identified (Herzog 1986). Finally, the molecule is secreted into the follicular lumen, where iodination of tyrosine residues of Tg occurs extracellularly, especially at the surface of the apical membrane of the thyrocyte. Sixty seven tyrosyls are present in monomeric Tg, and only some of the tyrosyls are available for iodination and hormonogenesis. Four major hormonogenic sites (a.a. 5, 1291, 2554, 2747), as well as three minor or limited ones (a.a. 513, 685, 2568) have been established (de Vijlder and den Hartog 1998, Lamas et al. 1989). Iodinated Tg re-enters the thyrocyte via receptor-mediated endocytosis and is enzymatically cleaved within lysosomes to generate free T4 and T3 that are eventually secreted into the extracellular space (Section 1.1). The iodine content of Tg and its function is discussed in detail in Section 1.3.

In the past two decades, diverse strategies have been used to search for pathogenic immunodominant T cell epitopes in Tg. One of them used mTg-specific, T-cell hybridoma clones as a tool to screen antigenic T cell epitopes in Tg (Champion et al. 1991, Texier et al. 1992). A second strategy used computerized algorithms to predict potential T-cell epitopes based on MHC binding (Verginis and Carayanniotis 2004). To date, several algorithms have been described for epitope prediction of various MHC class

I and II - binding peptides, based either on the analysis of natural MHC ligands or on the binding properties of synthetic peptides (Schirle et al. 2001). The selected candidate peptides were subsequently tested for their immunogenicity and pathogenicity in experimental animals.

So far, eight EAT-causing peptides have been identified in mTg through the use of the computerized algorithms (Carayanniotis et al. 1994, Chronopoulou and Carayanniotis 1992, Rao et al. 1994, Rao and Carayanniotis 1997, Verginis et al. 2002). Six out of the 8 epitopes (306-20, 1579-91, 1826-35, 2102-16, 2498-06, 2596-08) are A^k-restricted, one (2694-2705) is A^s-restricted and interestingly, the 9mer (2495-2503) is restricted by E^k but elicits EAT in mice of either k or s haplotype. The first EAT-causing peptide in hTg, the 40mer (1671-10), was identified using a MHC class I-restricted CTL hybridoma as a searching tool (Texier et al. 1992). Subsequently, two more pathogenic epitopes were identified in hTg: the 14 mer (2730-43) and the 20mer (2340-29). The former induced EAT in CBA ($H\text{-}2^k$) mice only after adoptive transfer of LNC that were primed in vivo with mTg and stimulated in vitro with this peptide (Hoshioka et al. 1993), and the latter contains several E^k-binding motifs and elicits EAT and T cell responses in AKR ($H\text{-}2^k$) mice (Karras et al. 2003). Studies in Tg fragments containing hormonogenic sites revealed two additional epitopes containing T4, T4 (5)(a.a.1-12) and T4 (2553)(a.a. 2549-2560), that cause thyroiditis in susceptible strains of mice (Hutchings et al. 1992, Wan et al. 1997). Of note, none of these epitopes has been characterized as immunodominant, since they cannot be readily detected by proliferative LNC assay following processing of intact Tg in vivo and/or in vitro (Carayanniotis 2003). Moreover, Flynn J. C. *et al.* (Flynn

et al. 2004b) have recently reported four human Tg peptides (a.a. 181-195, 418-432, 1518-1532, 2079-2093), which encompass naturally processed Tg epitopes and stimulated a proliferative response of Tg-primed splenocytes. Among these four peptides, only p2079 (a.a. 2079-2093) consistently mediated thyroiditis by both direct challenge with the peptides and adoptive transfer of Tg-primed, p2079-activated splenocytes.

TPO

Shortly after the discovery of Abs to Tg in HT, a second group of Abs against thyroid microsomal protein was identified in HT patients (Roitt et al. 1964, TROTTER et al. 1957). Later studies have found TPO accounts for virtually all the antigenic determinants recognized by microsomal Abs (Czarnocka et al. 1985, Kotani et al. 1986, Mariotti et al. 1989). Anti-TPO Abs are the most frequently represented A-Abs in the sera of patients suffering from AITD; they are present in 90% of HT and 74% of GD patients (Mariotti et al. 1990). These Abs can fix complement and are cytotoxic to thyroid epithelial cells in culture (Dawe et al. 1993). However, TPO is normally inaccessible to circulating Abs since it is present only on the apical portion of the follicular cells, suggesting that some prior damage to the tissue may be required before anti-TPO Abs can exert pathogenic effects in vivo (Khoury et al. 1984).

TPO is a membrane-bound, glycosylated, hemoprotein enzyme that plays a key role in the biosynthesis of thyroid hormones, iodination of Tyr residues on Tg, and intramolecular coupling of iodotyrosines to form T3 and T4. The primary structures of human (Kimura

et al. 1987, Libert et al. 1987), porcine (Magnusson et al. 1987), rat (Derwahl et al. 1989) and mouse (Derwahl et al. 1989, Kotani et al. 1993) TPO have been deduced from cDNA, and show considerable homology. The human TPO gene maps to the shorter arm of chromosome 2 and gives rise to two isoforms of protein through alternative splicing of the gene (Kimura et al. 1987). The longer protein, referred to as TPO-1, is 107 kDa in size and 933 a.a. in length; and the shorter one, referred to as TPO-2, lacks 57 a.a. (residues 533-589) in the middle of the sequence. The mRNA encoding TPO-2 is present in much less abundance than that for TPO-1. It has been long recognized that TPO gene transcription and translation are both under the positive control of TSH (Weetman 1990).

Immunization with thyroid microsome or purified TPO proteins has been used to induce thyroiditis in several models (Section 1.2.2.1). Epitope analysis of circulating TPO Abs has revealed > 90% of these Abs are directed against two conformational epitopes termed as immunodominant region A and B, but the Ab fraction within each individual serum directed against the A and B region varies from patient to patient (McLachlan and Rapoport 1995, Prummel and Wiersinga 2005). Particularly, epitope recognition did not differ in patients with GD vs HT, older vs younger, or hypothyroid versus euthyroid (Prummel and Wiersinga 2005). It was recently found that the key part of the B domain localizes between a.a 713 (Tyr) and 717 (Asp) of TPO (Bresson et al. 2004). Arg at position 225 and Lys at position 627 were reported as the key components of A and B domains (Gora et al. 2004), yet the exact location of domain A remains unclear.

Thyrotropin (TSH) receptor (TSHR)

TSHR is the major A-Ag in GD, in which thyroid-stimulating A-Abs (TsAb) activate TSHR and stimulate subsequent cAMP production by the thyrocytes (Schott et al. 2005). Although GD is characterized by the presence of TsAbs to TSHR, another group of patients with goitrous HT or atrophic thyroiditis show TSHR A-Abs that block thyroid activation, termed as thyroid-blocking Ab (TBAb), leading to hypothyroidism (Chiovato et al. 1994).

The TSHR belongs to the glycoprotein hormone receptor subfamily of G-protein-coupled receptors. The TSHR cDNA has a single open reading frame encoding a protein of 764 a.a, of which 21 a.a. is the signal peptide (Misrahi et al. 1990). The mature TSHR (without signal peptide) contains a heavily glycosylated amino-terminal ectodomain of 397 a.a., and a carboxyl terminal region divided into a 264-a.a. membrane-spanning segment and a cytoplasmic tail of 82 a.a. The single chain of TSHR on the cell surface is intramolecularly cleaved into disulfide-linked subunits A and B, with removal of 50 residues from the N-terminal of the B subunit (Davies et al. 2002). This TSHR cleavage is followed by the release of ligand-binding A subunit from the membrane-bound B subunit, referred to as receptor shedding (Couet et al. 1996). It was proposed that the shed A subunit, rather than the full-length receptor, is the crucial A-Ag in the generation of TsAb (Chen et al. 2003). It is suggested that the majority of TsAb epitopes are localized at the N-terminal and TBAb epitopes are at the C-terminal part of the extracellular

domain, despite the fact that TBAb and TsAb do have epitopes in close proximity (Schott et al. 2005).

1.2.4 Pathogenic mechanisms in the induction of AITD and EAT

Genetic influences on AITD and EAT

In humans, the cause of AITD is multifactorial, requiring interplay between genetic, endogenous and environmental factors. The association of human leukocyte Ag (HLA) genes with GD and HT (Weissel et al. 1980, Farid et al. 1981, Thompson and Farid 1985) has been reported in several epidemiological studies, but this linkage is relatively weak and inconsistent between populations due to ethnic diversity and linkage disequilibrium between DR and DQ genes. Recently, transgenic mice expressing certain HLA molecules have been generated to help clarify the role of HLA class II genes in HT susceptibility (Kong et al. 1996, Kong et al. 1997, Wan et al. 2002). EAT induction in these transgenic mice by immunization with human or mouse Tg indicated that mouse TcR can efficiently recognize the HLA-peptide complex, leading to T cell activation. These studies have shown that DR3 and DQ8, not DR2, DR4 and DQ6, were susceptible alleles to hTg immunization, whereas only mice bearing DR3, but not DQ8 allele developed EAT following immunization with mTg. Moreover, Flynn *et al.* have reported that coexpression of DR3 and DQ8 HLA molecule in MHC class II negative strain of mice significantly reduced the severity of mTg-induced EAT (Flynn et al. 2002).

Other genes, located outside of the HLA region, may also contribute to the genetic susceptibility of AITD. The cytotoxic T cell antigen-4 (CTLA-4) is one of strongest susceptibility genes that have been extensively studied in different populations. A single nucleotide polymorphism, a G allele at position CT60, in the 3' untranslated region of CTLA-4 has been associated with increase prevalence of GD in Japanese (Ban et al. 2005) and Italian (Petrone et al. 2005) populations. Furthermore, elevated serum levels of anti-TPO Ab is influcend by other single nucleotide polymorphisms in CTLA-4 promoter (-318 C/T) or encoding region (49 A/G in exon 1) (Zaletel et al. 2006). In addition, a CTLA-4 microsatellite polymorphisms (allele 106) was shown to be significantly increased in patients with GD or HT in both USA and UK studies (Kotsa et al. 1997, Yanagawa et al. 1995). Interestingly, Tomer and his colleagues (Tomer et al. 2002) have reported that a new microsatellite inside intron 27 of the Tg gene was strongly linked to AITD in Caucasian patients. In two recent studies, a C/T polymphism in Kozak sequence of CD40, a costimulatory molecule expressed on APC, have also been liked to GD and/or EAT susceptibility in mice (Jacobson et al. 2005) and humans (Ban et al. 2006).

In induced murine models of thyroiditis, the type and extent of disease are heavily influenced by the MHC haplotype of the animals. In one study (Vladutiu and Rose 1971a), 33 inbred mouse strains representing 11 different haplotypes were used and were classified into excellent ($H\text{-}2^{k,\ s}$), good ($H\text{-}2^{q}$), fairly good ($H\text{-}2^{a,\ m,\ p}$), poor ($H\text{-}2^{b,\ d}$) and very poor ($H\text{-}2^{v}$) responders based on the existence of thyroid infiltration and the extent of its follicular destruction. By using intra-H-2 recombinant mouse strains derived from recombination of $H\text{-}2^{k,d,b}$ alleles, the major control gene for murine EAT was localized to

the I-A locus (Beisel et al. 1982a, Tomazic et al. 1974). The importance of this major control gene was confirmed in mice that have been treated with anti-I-A mAbs prior to or at the time of Tg/CFA challenge, which failed to develop EAT (Vladutiu and Steinman 1987). Moreover, the genetic restriction of EAT induction varies between immunizing Ags. In the TPO-induced model, mice bearing the H-2^b haplotype were good responders and H-$2^{a,d,k,s}$ strains were not (Kotani et al. 1990, Vladutiu and Rose 1971a). On the other hand, the H-$2^{d,u}$ haplotypes were susceptible to TSHR-induced thyroiditis, whereas the H-2^b and H-2^k haplotypes were resistant (Many et al. 1996). Class I MHC genes were also suggested to modulate the severity of the pathogenic autoimmune responses in animals. For instance, B10.D2 mice, with a D^d gene, showed a markedly reduced cellular infiltration than congeneic strains with D^k or D^f allele (Kong et al. 1979). Other studies using strains of mice with point mutations at the H-2K gene or intra-H-2 recombinant mice showed that mice bearing K^k developed less EAT than those with K^q or K^b, suggesting that K-end genes can also modify the responses (Maron and Cohen 1979, Maron and Cohen 1980). Similarly as in human studies, non-MHC genes were also reported to influence EAT susceptibility, using congeneic mouse strains carrying the same H-2 but different background genes (Beisel et al. 1982b, Verginis and Carayanniotis 2004).

T-cell mediated injury

T cells have been considered to be critical for the induction of EAT, not only as effector cells, but also as helpers to activate B cells. The influence of MHC haplotype, as

discussed above, indirectly suggests T cells are involved in the development of EAT. Furthermore, histological examination revealed that T cells were the dominant subset in thyroids of mice that developed Tg-induced EAT (Lillehoj and Rose 1982). The direct evidence for the role of T cells came from the induction of EAT in naïve recipients by adoptive transfer of Tg-primed LNC or splenic cells (Braley-Mullen et al. 1981, Okayasu 1985, Simon et al. 1986) or Tg-specific T-cell clones or lines (Maron et al. 1983, Romball and Weigle 1987). Lastly, nude mice that lack mature T cells failed to develop EAT after challenge with either Tg emulsified in CFA (Vladutiu and Rose 1975) or soluble Tg followed by LPS injection (Esquivel et al. 1977).

The role of T cell subsets in pathogenesis of EAT has also been investigated using depleting anti-CD4 and anti-CD8 Abs. Flynn et al. (Flynn et al. 1989) reported that $CD4^+$ populations were the primary cells mediating the initial transfer and development of the disease in syngeneic recipients. $CD8^+$ T cells probably exert cytotoxic effects in the late stages, which increased the severity of disease upon cotransfer with $CD4^+$ cells. The role of $CD4^+$ T cells as EAT inducers was confirmed in a later study (Stull et al. 1988), in which splenocytes isolated from mice treated with anti-CD4 mAb before receiving Tg did not proliferate in vitro to Tg and were unable to transfer disease to naïve mice. Furthermore, $CD8^+$ CTL were demonstrated to be involved in thyroid destruction in vivo (Creemers et al. 1983, Simon et al. 1986) and in vitro (Kong et al. 1986, Salamero and Charreire 1985). Interestingly, injection of anti-CD8 Ab into recipient mice facilitated the induction and persistence of granulomous thyroiditis induced by transfer of mTg-primed

splenic cells (Braley-Mullen et al. 1994), suggesting $CD8^+$ T cells may be required for the resolution of granulomous thyroiditis.

Many efforts have been devoted to study the process of initial activation of thyroid-specific autoreactive T cells. The discovery of MHC class II expression on thyroid cells in AITD (Bottazzo et al. 1983, Hanafusa et al. 1983) raised the hypothesis that aberrant MHC expression in the thyroid could initiate or perpetuate the autoimmune response. Further work demonstrated that IFN-γ is the only cytokine capable of inducing class II expression on thyrocytes in vitro (Todd et al. 1985, Weetman et al. 1985), and class II expression in vivo always follows the appearance of infiltrating lymphocytes which secrete IFN-γ (Hamilton et al. 1991, Margolick et al. 1988). Recently, Kimura *et al.* (Kimura et al. 2005) generated transgenic mice that express MHC class II molecules in the thyroid to examine the role of MHC^+ thyrocytes in the induction of EAT. They found that MHC class II expression on thyrocytes alone did not cause spontaneous thyroiditis, but mildly increased its severity following immunization with Tg. Furthermore, it has been reported that CD40 is expressed on thyroid follicular cells in both healthy individuals and patients, and is upregulated by IL-1α and IFN-γ (Metcalfe et al. 1998). Based on these observations, Weetman proposed that autoreactive T cells could be activated in AITD through their CD40L binding to CD40 expressed on thyrocytes (Weetman 2003).

B-cell mediated injury

Exactly how the autoimmune response interferes with thyroid function is unclear, but it seems likely that both T- and B- cell mediated mechanisms are involved. Development of A-Abs to thyroid Ags, Tg, TPO and TSHR, is one of the most common features of AITD, and is used as a diagnostic criterion in humans. Early studies reported that passive transfer of immune sera containing anti-Tg Abs instigated mild disease in mice (Weetman 2003, Tomazic and Rose 1975) and rabbits (Vladutiu and Rose 1971b), but this was not confirmed in other studies (Rose et al. 1973, Okayasu 1985). Moreover, there was no correlation between Ab titers and severity of disease (Esquivel et al. 1978, Vladutiu and Rose 1975). Lastly, mice depleted of B cells by treatment from birth with anti-IgM developed EAT following Tg immunization with lower incidence (63% vs 88%) and less severity (Rayfield et al. 1989, Vladutiu 1989). Although B cell depletion is not complete in these animals, these findings suggest B cells may not be necessary for EAT induction, but can increase its severity and prevalence. This has also been confirmed in a model of SAT, in which NOD.H-2h4 mice genetically deficient in B cells (NOD.Kμ^{null}) or treated by anti-IgM developed minimal SAT (Braley-Mullen and Yu 2000).

B cells themselves can contribute to the pathogenesis of AITD as professional APC, or effector cells that produce Abs mediating complement-mediated damage, Ab-dependent cell-mediated cytotoxicity (ADCC) or direct alteration of thyrocyte function (Stafford and Rose 2000). In adult NOD.Kμ^{null} mice as mentioned above, passive transfer B cells or anti-Tg A-Abs did not render them susceptible to SAT, suggesting B cells were required

for the early activation of $CD4^+$ T cells, functioning either as important APC for T cell activation or to amplify responses of effector T cells (Braley-Mullen and Yu 2000). Deposition of immune complex or membrane attack complexes has been identified in the thyroids of HT and GD patients (Weetman and McGregor 1994). When anti-Tg Abs were passively transferred into mice, immune complexes containing Tg and its Abs were found in the follicular basement membrane, followed by neutrophil infiltration (Clagett et al. 1974). These findings suggest a role for complement-mediated destruction induced by immune complexes in the thyroid gland. ADCC has been demonstrated using normal lymphocytes as effectors and thyroid cells incubated with HT or normal sera as targets (Bogner et al. 1984). Similar findings have been observed in guinea pig and OS chicken, where Abs from diseased animals rendered normal lymphocytes cytotoxic for Tg-coated erythrocytes (Ringertz et al. 1971, Wick et al. 1982). Abs can also participate in AITD by directly modifying cell function, such as TsAb and TBAb mentioned above.

Other mechanisms

Other mechanisms, such as Fas-mediated apoptosis and cytokines (IFN-γ and IL-12), have also been studied in AITD. Due to the absence of reliable anti-CD178 (FasL) Abs in earlier studies, controversial results have been reported. Some authors reported both CD95 and CD178 were present in glands from HT patients but CD95 was absent in the normal thyrocytes, whereas others showed that CD178 but not CD95 was absent in the normal thyrocytes (reviewed in (Stassi and De Maria 2002)). However, there is general agreement that both of them are upregulated and simultaneously expressed on thyrocytes

in HT (Stassi and De Maria 2002). It has been demonstrated that expression of CD178 in the thyroid, encoded by either plasmid DNA (Batteux et al. 1999) or transgene (Batteux et al. 2000), induced cell death of infiltrating T cells, therefore, exerting a protective role by limiting the development of EAT. Conversely, interactions between CD95 and CD178 were reported to mediate thyrocyte destruction in HT but not GD by autocrine or paracrine killing (Stassi et al. 2000).

The contribution of IL-12 and IFN γ to EAT has been examined using several approaches. Administration of anti-IL-12 neutralizing Abs, particularly at the time of priming in CBA/J mice, significantly suppressed the development of EAT; while injection of recombinant IL-12 enhanced it (Stafford and Rose 2000). Moreover, IL-12 p40 knockout mice developed very little thyroiditis following immunization with mTg/CFA (Zaccone et al. 1999). In contrast to the crucial requirement for IL-12 in instigating and accelerating early autoimmune processes in EAT, the role of IFN-γ in EAT is complex and has not yet been clearly defined. Systemic delivery of IFN-γ (Kawakami et al. 1990) or anti-IFN-γ Abs (Tang et al. 1993) has been shown to facilitate or prevent EAT induction, respectively. However, addition of anti-IFN γ mAbs during in vitro activation of mTg-primed splenic cells resulted in more severe EAT in recipient mice upon transfer (Stull et al. 1992). Using knockout mice that lack IFN-γ or its receptor, it has been suggested that deficiency in IFN-γ signaling may ameliorate (Yu et al. 2002) or do not affect (Tang et al. 1998, Alimi et al. 1998) EAT induction. Two other studies have reported tha thyroid-specific expression of IFN-γ transgene can either induces hypothyroidism in C57BL/6 mice (Caturegli et al. 2000), or limits SAT in

NOD.H-2h4 mice (Barin et al. 2003). Recently, Yu *et al.* showed that splenic cells or bone marrow isolated from IFN-$\gamma R^{-/-}$ NOD.H-2h4 mice could not transfer disease to either IFN-$\gamma R^{-/-}$ or WT mice (Yu et al. 2006a), suggesting that thyrocytes responding to IFN-γ may be essential for development of SAT. Other cytokines, such as TNF α (Chen et al. 2006), are also involved in the induction of EAT.

1.3 THE ROLE OF IODINE IN AITD

1.3.1 Variations of iodine content in Tg

Iodine is not only an essential trace element for synthesis of Tg and thyroid hormones, but also directly influences most of the thyroid-specific functions as well as thyrocyte growth. The adult human body contains 15 -20 mg of iodine and roughly 80% is located in the thyroid gland, mostly in the form of Tg (Underwood 1977). The recommended daily iodine intake is variable, depending on the age of the subject: 40 μg/day during the neonatal period and 150 μg/day in the adult (Roti et al. 1997). Also, iodine requirement increases to approximate 200 μg/day during pregnancy. In the United States, the average iodine intake is approximately 200 μg daily and may reach as much as 500 μg. As summarized in Pennington's review (Pennington 1990), it was proposed by many studies that an iodine level $\leq$ 1.0 mg per day was the safe upper limit for adults.

The thyroid gland is able to actively take up and accumulate iodide by a factor of 20- to 40- fold with respect to its concentration in the plasma (Carrasco et al. 2000). Inorganic iodide actively enters the thyroid follicular cells and is incorporated into tyrosine residues on Tg through a series of metabolic steps, forming thyroid hormones (Section 1.1). It is well established that iodination of Tg in the thyroid is a posttranslational event, which probably occurs on fully aggregated Tg rather than on its subunits. Most of Tg in the normal gland is present in the follicular lumen. However, small amounts of Tg could enter the circulation either by secretion of newly synthesized Tg through the basolateral

membrane (Chambard et al. 1987, Schneider et al. 1983), or transcytosis of stored Tg from the lumen (Herzog 1983). Transcytosis must occur at a very low rate since most of the endocytosed Tg is degraded intracellularlly to release T3 and T4 hormones.

Tg purified from thyroid tissues varies in total iodine content, depending on the amount of iodine intake. For example, normal human Tg purified in a soluble form varies widely in iodine content from as low as 0.1% to as high as 1.1%, which corresponds to about 10-50 atoms of iodine per mole of Tg (Taurog 2000). However, it has been reported that the insoluble fraction of luminal Tg is highly iodinated but contains no hormones (Herzog et al. 1992, Berndorfer et al. 1996, Baudry et al. 1998). In contrast, serum Tg contains little or is completely devoid of iodine in normal individuals, because it arises, most likely, from poorly-iodinated newly-synthesized Tg (Ikekubo et al. 1981, Schneider et al. 1983). Yet, in patients with some thyroidal abnormalities, such as GD and thyroid cancers, increased iodine content in circulating Tg was reported (Druetta et al. 1999). Interestingly, administration of TSH in rats was shown to increase the iodine content of circulating Tg to a level similar to intra-thyroidal Tg, suggesting that TSH may facilitate the release of previously synthesized, iodinated and stored Tg (Schneider et al. 1985).

1.3.2 Iodine content of Tg alters its immunogenicity

Iodine content in Tg modifies its structure via oxidation of –SH groups and/or substitution in the phenolic ring of tyrosyl residues (Edelhoch et al. 1969), Tg molecules

with different iodine contents adopt different conformational shapes as assessed by electron microscopy (Berg and Ekholm 1975). Furthermore, highly iodinated Tg (HI-Tg) is less susceptible to hydrolysis by thyroid acid protease (Lamas and Ingbar 1978). Taken together, the changes in 3-D structure and proteolysis susceptibility by iodination imply that iodine may alter the immunogenic properties of Tg.

Indeed, iodination of Tg alters its immunogenicity at both T- and B-cell level, as well as its pathogenicity. An earlier study demonstrated that excess iodine intake in CS chicken causes a significant increase in iodine content in purified Tg, from less than 13 iodine atoms per molecule (NI-Tg) to higher than 60 iodine atoms per molecule (HI-Tg) (Sundick et al. 1987). HI-Tg induced stronger Ab responses to HI-Tg, T3 and T4 in normal K strain chickens, in comparison to Tg depleted of iodine by propylthiouracil (LI-Tg). However, no Ab was detected against LI-Tg in either HI-Tg- or LI-Tg- immunized chickens. In NB and/or BB Wor rats, treatment with NI-Tg but not LI-Tg - generated by methimazole, induced anti-Tg Abs and EAT (Ebner et al. 1992). Similar findings were also reported in humans, as Tg iodinated in vitro by iodobeads (150 I atoms per molecule), were recognized differentially by anti-Tg mAbs, as compared to NI-Tg, and LI-Tg purified from a patient with non-toxic goiter (Saboori et al. 1998). The above studies have indicated that the iodine content in Tg can modify its B-cell stimulatory capability, which probably results from the 3-D structural change during modification.

At the T-cell level, Allen *et al.* compared the relative frequency of precursor T cells against HI-Tg, NI-Tg or LI-Tg in the LT-prone BB/W rats, and found no difference in splenic T lymphocytes (Allen and Thupari 1995). These results suggest that the difference in the immunogenicity of Tg with variable iodine content is not due to variations in the number of T cells recognizing these Tg preparations. A critical role of iodination for T cell recognition was initially highlighted in studies using Tg peptide containing hormonogenic sites. Champion and coworkers first identified two Tg-specific T-cell hybridomas that recognize only Tg with sufficient iodine content (> 1 T4 per molecule), but not LI-Tg obtained from mice treated with the TPO inhibitor 3-amino-1,2,4-triazole (Champion et al. 1987). Using these T-cell hybridomas, they identified an EAT-causing peptide which contains thyroxine, T4 (2553) (Champion et al. 1991). Later studies have compared peptide T4 (2553) and its thyronine (T0) –containing analog, and found both T0 (2553) and T4 (2553) peptides were able to stimulate Tg-primed LNC and prime thyroditogenic T cells in vivo (Kong et al. 1995b). Subsequently, Kong identified another Tg pathogenic peptide containing a hormonogenic site at position 5, and immunization with either T4(5) or T0(5) elicited similar EAT (Wan et al. 1997). These findings suggest iodine residues on the outer ring of L-thyronine are not necessary for autoreactive T cell activation and EAT induction.

In a recent study, Rasooly and coworkers described for the first time that recognition of Tg by peripheral human lymphocytes depends on its iodination content (Rasooly et al. 1998). Lymphocytes purified from normal individuals or HT patients showed

proliferation in vitro to NI-Tg and HI-Tg, but not LI-Tg. Iodination of LI-Tg increased its immunogenicity to stimulate lymphocyte proliferation. Our laboratory has also reported that HI-Tg is highly immunopathogenic in SJL mice – a strain which does not develop spontaneous thyroiditis – causing EAT of higher incidence and severity as well as stronger B- and T-cell responses than those elicited by NI-Tg (Dai et al. 2002). In vitro experiments demonstrated that altered processing of HI-Tg in APC can generate a cryptic pathogenic peptide (2495-2503) (Dai et al. 2002), which allowed us to postulate that Tg iodination may promote generation of pathogenic epitopes to which immune tolerance has not been previously established.

1.3.3 Effect of excess iodine on thyroid function

Wolff-Chaikoff effect (Acute inhibitory effect of iodine)

Administration of small to moderate amounts of iodide to rats and/or humans enhances intrathyroidal iodine levels, resulting in augmented formation of the thyroid hormone (Nagataki and Ingbar 1964). However, when large amounts of iodide were given, organic binding of iodine was inhibited in the gland. The decreasing yield of organic iodine from increasing doses of inorganic iodide is termed the "acute Wolff-Chaikoff effect" (Wolff and Chaikoff 1948). In cultures of bovine or dog thyroid slices, this acute inhibition effect was reported to be caused by diminished generation or decreased availability of H_2O_2 in the thyroid, and can be prevented in the presence of TSH or a H_2O_2-generating system (Corvilain et al. 1988, Taurog 1970).

The Wolff-Chaikoff effect induced by a single dose of iodide is only a transient phenomenon, as intrathyroidal organification of iodine resumes in about 26-40 hours after iodide administration (Wolff et al. 1949). This "escape" or "adaptation" was shown by Braverman and Ingbar to be related to the decreased activity of the iodide-transport system, which lowered the intrathyroidal iodine concentration (Braverman and Ingbar 1963). Later studies revealed that decreased expression of NIS, possibly TPO, in the thyroid attributes to the adaptation (Eng et al. 1999, Uyttersprot et al. 1997, Ferreira et al. 2005). This inhibition of iodide transport prevents the development of hypothyrodism or goiter in normal thyroid from humans or animals given excess iodide. However, during chronic exposure to excess iodide, the adaptation is not complete in certain subjects, and the quantity of iodine accumulated and organified is well higher than normal (Sundick et al. 1987).

Direct toxic effect of iodine

Excess iodide exerts effects on many aspects of the thyroid, including its growth in vivo and cell proliferation in vitro (Pisarev 1985). Naturally occurring iodine toxicity in normal individuals is rare, and supplemental iodide is added to virtually all animal diet to ensure iodine adequacy. Yet, certain populations who consume large quantities of seaweed or kelp, or patients exposed to therapy with iodine-containing medications, are at greater risk for iodine toxicity.

In animals with thyroid hyperplasia induced by a low iodine diet, oral administration of large doses of iodine induced necrosis of thyroid follicular cells as early as 5.5-6 hrs after administration (Belshaw and Becker 1973, Mahmoud et al. 1986). Mahmoud *et al.* also suggested in their study that necrosis was due to direct interaction of iodide with the epithelial cells, and not by iodide-induced vasoconstriction or increase in TSH level. This direct toxic effect of iodide on thyrocytes was also observed in autoimmune-prone animals on iodide-supplemented diet, such as BB/W rats (Li and Boyages 1994), OS chickens (Bagchi et al. 1985) and NOD mice (Many et al. 1995). The affected cells often had swollen and disrupted mitochondria, marked accumulation of secondary lysosomes and lipid droplets, extreme dilation of rough endoplasmic reticulum, ruptured luminal cell membrane, as well as clumping of chromatin in the nucleus, indicating cell necrosis. The above observations were confirmed in vitro in cultured human thyroid follicles (Many et al. 1992). Follicles incubated in the presence of high concentrations of iodide (10^{-3} to 10^{-5} M) had a significant increase in percentage of necrotic cells, as compared to those grown in the presence of low iodide (10^{-7} M). Thyroid cell injury may create an inflammatory environment, thus serving as an initial event in iodide-induced AT in these animals.

1.3.4 Iodide-induced hypothyroidism and/or goiter

Many individuals are exposed to large quantities of iodine and/or iodide present in proprietary medications, in food as preservatives, and in x-ray contrast media. Usually, excess iodine is well tolerated by healthy individuals because of escape from its acute inhibitory effect on the organification mechanisms (Wolff-Chaikoff effect). Yet, in

certain subjects with susceptible genetic background or underlying thyroid dysfunction, exposure to large doses of iodine and/or iodide usually results in hypothyroidism and/or goiter (Pennington 1990, Markou et al. 2001).

Iodide-induced hypothyroidism and/or goiter in humans

In patients with underlying or previous thyroid disease

Thyroid disease is commonly associated with underlying defects in the intrathyroidal organic binding of iodide (Vagenakis and Braverman 1975), thus predisposing such patients to develop iodide-induced hypothyroidism. Patients with HT frequently develop hypothyroidism as a result of the chronic autoimmune destruction of the thyroid. In euthyroid patients with HT, the administration of ~ 180 mg of KI daily induced hypothyroidism in 60% of the patients after 4 to 5 weeks (Braverman et al. 1971). The same regimen also led to a prompt and sustained decrease in serum T4 and a striking rise in TSH in all patients with diffuse goiter previously treated with ^{131}I (Braverman et al. 1969). Furthermore, long-term (60-120 days) administration of pharmacological quantities of iodide (300-350 mg KI) have been shown to induce hypothyroidism in patients with a previous history of thyroid disease, including subacute thyroiditis (Roti et al. 1990), post-partum thyroiditis (Roti et al. 1991) and IFNα-induced thyroid disfunction (Minelli et al. 1997). All subjects in the above studies had recovered from thyroid diseases, and had a normal range of baseline TSH before iodide administration. However, undetectable abnormalities in thyroid function may persist in these patients and account for the iodide-induced hypothyroidism.

In patients without apparent thyroid disease

Iodine is readily transferred across the placenta and is actively transported and secreted by breast tissue into the milk (Roti et al. 1983). Iodide-induced goiter and/or hypothyroidism has also been reported in the fetus or newborns when pregnant or nursing mothers have received large quantities of iodine (Wolff 1969, De Wolf et al. 1988, Pennington 1990, Vicens-Calvet et al. 1998). In order to study the effects of iodide during perinatal life, pregnant or nursing Sprague-Dawley rats were fed with either tap water or water containing 0.01% NaI. The term fetus and neonatal rats from NaI-treated mothers developed hypothyroidism, but the thyroid function returned to normal from 18 to 60 days of age in spite of continued iodide administration (Theodoropoulos et al. 1979). The findings in rats suggest that resistance mechanisms to the inhibitory effect of iodide on thyroid hormone synthesis are probably developed after birth.

Patients with chronic nonthyroidal illnesses do not generally manifest thyroid dysfunction. However, a few cases of such patients were reported to develop iodide-induced hypothyroidism. This condition has been observed in adult patients with chronic renal failure (Sato et al. 1992), chronic respiratory disease treated with iodine (OLINER and RUBINSTEIN 1957, OPPENHEIMER and McPHERSON 1961), or children with cystic fibrosis treated with sulfisoxazole (Azizi et al. 1974). These patients do not have an apparent accompanying thyroid disease, but the failure of other organs, such as kidney, may interfere with the metabolism of iodine, thus rendering the patients susceptible to iodine excess.

Subclinical hypothyroidism, defined as elevated serum TSH but normal T3 and T4 values, has been detected in healthy school children in rural China, where moderate or excessive iodine was taken from water or salt (median urinary iodine= 338 or 631 μg/L) (Gao et al. 2004). Thyroid A-Abs were negative in all except one of the patients. Similar results were obtained in elder residents in iodide-rich regions in Hungary and Denmark (Szabolcs et al. 1997, Laurberg et al. 1998). Although the prevalence of subclinical hypothyroidism was significantly higher in high-iodide intake regions, the levels of serum A-Abs was similar to, or even lower than, those in a low-iodide region. Interestingly, iodide-induced hypothyroidism and/or goiter were also observed in apparently healthy adults. Increased prevalence of endemic goiter has also been reported in Japanese populations consuming the iodine-rich seaweed called kombu (Suzuki et al. 1965) and in Chinese communities consuming high iodine (Li et al. 1987, Zhao et al. 1998, Teng et al. 2006). All goitrous patients in these studies were clinically euthyroid. Other studies have shown serum concentration of TSH and thyroid hormones are associated with the amount of iodide intake, individuals with excess iodide intake are more susceptible to develop hypothyroidism (Konno et al. 1993, Konno et al. 1994, Khan et al. 1998, Vagenakis et al. 1973). Konno *et al.* demonstrated that the presence of thyroid A-Ab was not associated with hypothyroidism, however when the iodine intake was restricted, the increased TSH levels returned to normal in patients without antithyroid Abs but remained elevated in those with Abs (Konno et al. 1993). This finding suggests iodide-induced hypothyrodism is transient and may result from inhibition of organic iodine formation, but autoimmune-based hypothyroidism in HT persists even after iodide withdrawal (Konno et al. 1993, Konno et al. 1994, Khan et al. 1998, Vagenakis et al. 1973).

In all human studies mentioned above, it remained inconclusive to what extent the effects of iodide had an autoimmune basis, since the antithyroid Abs were either untested or remain unrelated to the development of hypothyroidism. Usually, iodide-induced hypothyrodism is reversible, and thyroid function usually returns to normal when iodide is withdrawn (Konno et al. 1993, Vagenakis et al. 1973). The mechanisms underlying these phenomena remain unclear but it is postulated that they may involve inhibitory effects of iodine excess on: a) iodide organification, i.e. an inability of some subjects to escape a persistent Wolff-Chaikoff effect, or b) the release of T4 or T3 from the thyroid.

Iodide-accelerated thyroiditis and/or hypothyroidism in animals

The effect of iodide administration on the development of EAT is largely dependent on thyroid function and genetic susceptibility of the experimental animals. In animals without underlying thyroid dysfunction, such as W-line and Wistar rats (Allen et al. 1986), Biozzi and CBA/J mice (Hutchings et al. 1999, Braley-Mullen et al. 1999), no thyroiditis was induced after long term exposure to an iodine-rich diet. Indeed, thyroid autoimmunity, assessed by the production of anti-Tg Abs, was depressed in Wistar rat after iodine administration (Mooij et al. 1994a). However, when animals were simultaneously immunized with thyroid A-Ag, such as thyroid extract, treatment with excess iodine exacerbated the development of EAT (Evans et al. 1969).

In 1985, the role of excess iodine on autoimmune-prone animals was first examined in chickens (Bagchi et al. 1985). Newly hatched CS chickens, a strain spontaneously

developing low incidence of LT, were supplied with normal water or water containing KI. By 10 weeks, the degree of lymphocytic infiltration of the thyroid glands was significantly increased in iodide-treated chickens, in a dose-dependent manner, accompanied by an increase in Abs against Tg, T3 and T4 Abs. The related OS chickens, highly susceptible to LT, were used to determine whether iodine-deficient regimens could prevent or reduce thyroid A-Ab responses and thyroiditis. Administration of T4 in the presence of iodine transport inhibitor (KCLO4) caused a significant reduction in the production of anti-Tg, T3 and T4 Abs.

Three groups have demonstrated that long-term iodide administration (up to 12 weeks) significantly increased the incidence of LT in young BB/W rats (Allen et al. 1986, Li et al. 1993, Mooij et al. 1993). However, thyroid weight and serum T3, T4, TSH and anti-Tg Ab concentration were not affected by iodine treatment. Of note, the focal infiltrations in the diseased glands started with increases in numbers of MHC class II-positive dendritic cells (DC), suggesting iodine may mediate this autoimmune process by stimulating APC, thus activating T and B cells. In NOD mice, dietary iodine increased the incidence and severity of the thyroid lesions although A-Abs to Tg were absent (Hutchings et al. 1999). Yet, both thyroiditis and anti-Tg Abs were accelerated in similarly treated NOD-H-2^{h4}, which express I-A^{k} on the NOD genetic background (Rasooly et al. 1996, Hutchings et al. 1999, Braley-Mullen et al. 1999). Histological analyses of the thyroids have shown that following 1-week of iodide administration, MHC class I expression is elevated on thyroid follicular cells and T cells have begun to infiltrate (Verma et al. 2000). Similarly, i.p.

injection of iodide elicited thyroiditis in hamsters with thiouracil – induced hyperplastic goiter (Follis R.H. 1964).

In most of the above studies, the effect of iodine on thyroid function remained unclear, since the concentration of serum thyroid hormones were either not examined (Rasooly et al. 1996, Bagchi et al. 1985, Hutchings et al. 1999, Braley-Mullen et al. 1999) or found unchanged despite the accelerated EAT (Allen et al. 1986). To mimic the situation in humans with thyroid dysfunction, hemithyroidectomy was performed in rats at 30 days of age before they were placed on NaI-supplemented or control water. At 90 days of age, it was found that iodine excess did induce thyroid enlargement in the residual lobe and hypothyroidism (↑TSH, ↓T3, ↓T4) in both autoimmune-prone BB/w rats and non autoimmune-prone W-line rats (Allen et al. 1986), regardless of the development of EAT. This regimen strikingly increased the incidence of spontaneous LT in iodide-treated rats (68% vs 13%) in BB/W rats, but did not induce LT in W-line, Wistar-Furth, or Sprague-Dawley rats. Similar findings were reported in NTx- Buffalo rats, which had an increased incidence of spontaneous LT over normal Buffalo rats (Allen and Braverman 1990). Iodide administration significantly increased the incidence of LT in these rats from 31% in the control group to 73% in the iodide-treated group ($p<0.05$). Serum TSH concentrations and anti-Tg Ab titers were also significantly higher in the iodine-treated rats. Changes in thyroid function have also been reported in dogs fed a high iodine commercial diet (Castillo et al. 2001). The daily iodine intake of these dogs from food

varied from 400-600 μg to 2.58-3.75 mg of KI. Dogs fed a high iodine diet had significantly lower total and free T4 and increased TSH in their serum.

After considering the observations in both humans and animals, one may conclude that iodine contributes to the development of hypothyroidism via two independent mechanisms: 1) exacerbating preexisting autoimmune thyroiditis via an autoimmune mechanism; 2) exerting prolonged inhibitory effects on iodide organification, preventing the synthesis or release of thyroid hormones; 3) affecting the thyroid structure and/or hormone synthesis through direct tissue damage.

1.4 DENDRITIC CELL, CELL DEATH AND AUTOIMMUNITY

1.4.1 Ag presentation by dendritic cell (DC)

DC are professional APC, unique in their capacity to maintain self tolerance and initiate primary immune responses. DC exist in two functionally and phenotypically distinct states according to their developmental stage: immature and mature. Tissue resident DC are most likely at an immature stage, and are very efficient in Ag internalization (Guermonprez et al. 2002). Pathways employed by DC for Ag capture include: 1) receptor-mediated endocytosis using C-type lectin receptors, Fc receptors, complement receptors, or scavenger receptors; 2) Phagocytosis of particulate Ags, such as pathogens, apoptotic and necrotic bodies; and 3) Macropinocytosis of soluble Ags (Guermonprez et al. 2002). Immature DC express low levels of surface MHC class I, class II and little costimulatory molecules (CD80, CD86, CD40), and are insufficient for T cell priming. Under steady-state non-inflammatory conditions, presentation of self Ags by tissue resident DC in the absence of costimulation leads to clonal T cell anergy, which is critical for the maintenance of peripheral tolerance (Wilson et al. 2004). In order to acquire the capability of T cell stimulation, DC must undergo a characteristic process of terminal differentiation called maturation. Mature DC are characterized by decreased capability of Ag intake, and upregulated surface expression of MHC and costimulatory molecules, as well as secretion of large amounts of proinflammatory cytokines (Banchereau et al. 2000). These modifications facilitate the formation and transportation of MHC/peptide complexes to the cell surface, increasing the T cell priming ability of DC. Maturation is also associated with a rapid migration of Ag-bearing DC from the inflamed tissues to the

T cell zone of secondary lymphoid organs, where they encounter and activate Ag-specific naïve T cells.

Maturation stimuli differ in their ability to educate DC to activate Th1 or Th2 cells, or conversely regulatory T cells (Treg). DC matured by pathogen-related molecules such as LPS (Rescigno et al. 1999), CD40L (Cella et al. 1996), oligo CpG nucleotides (Hartmann et al. 1999) or double stranded RNA (Cella et al. 1999) produce high levels of IL-12 and preferentially induce Th1 responses. On the other hand, stimuli like PGE2, TGF-β, β2-agonists (Kalinski et al. 1999), or OX40L (Liu et al. 2001b) inhibit secretion of IL-12 by DC and promote a Th2-polarized priming. Interestingly, Lutz *et al.* found semi-mature DC treated with TNF-α expressed high levels of MHC and costimulatory molecules but secreted little IL-12 (Menges et al. 2002, Lutz and Schuler 2002). Administration of TNFα-treated DC induced tolerance mediated by $CD4^+$ $IL\text{-}10^+$ T regulatory cells (Menges et al. 2002, Verginis et al. 2005). As revealed by the above studies, production of proinflammatory cytokines, especially IL-12, by DC, seems to be a critical factor in determining the outcome of T-cell stimulation.

Besides activating naïve T cells, DC can also directly activate naïve and memory B cells. It was reported that DC can retain unprocessed Ags in vitro and in vivo, and transfer these Ags to naïve B cells to initiate a specific Th2-associated Ab response (Wykes et al. 1998). As reviewed in (Banchereau et al. 2000), DC not only induce surface IgA expression on CD40-activated naive B cells and help them differentiate into plasma cells, but also

enhance the differentiation of CD40-activated memory B cells towards IgG-secreting cells.

1.4.2 DC-mediated induction or inhibition of autoimmunity

Participation of DC in autoimmune diseases was first indicated by the presence of DC in autoimmune lesions in both humans and animals. It was found DC are among the first infiltrating cells in the thyroid and pancreas in BB rats and NOD mice that spontaneously develop diabetes and thyroiditis (Canning et al. 2003, Turley 2002), as well as in rheumatoid synovial fluid and synovial tissue in patients with rheumatoid arthritis (Thomas et al. 1994). Furthermore, DC, surrounding islets in NOD mice, have been shown to acquire Ag and probably present them to T cells in local draining lymph nodes (Clare-Salzler and Mullen 1992, Shimizu et al. 1995).

The important role of DC as APC in initiation of autoimmunity has been revealed by transfer of A-Ag-pulsed DC into susceptible animals. Induction of Tg-loaded DC has been discussed in Section 1.2.2.2. In 1999, another study showed that adoptive transfer of DC expressing pathogenic self-peptides from myelin basic protein (MBP), Ac1-11, in combination with Ac1-11-specific transgenic $CD4^+$ T cells, were able to induce development of experimental autoimmune encephalomyelitis (EAE) in B10.PL mice (Dittel et al. 1999). Later studies demonstrated that DC pulsed with self peptide, such as myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (Weir et al. 2002) or cardiac

α-myosin peptide 614–629 (Eriksson et al. 2003), could induce organ-specific autoimmune disease, EAE or myocarditis, respectively, in susceptible mice, without cotransfer of peptide-specific T cells. Interestingly, in transgenic mice expressing lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) in pancreatic β islets or β-galactosidase in cardiomyocytes, repetitive immunization with DC constitutively expressing LCMV-GP immunodominant epitopes or DC propagated in vitro with β-galactosidase peptide, induced development of autoimmune diabetes, arthritis and myocarditis, respectively (Ludewig et al. 1998, Ludewig et al. 2000). Taken together, the above results indicate that splenic DC or BM-DC, loaded with self Ags, could activate naïve autoreactive T cells in vivo, thus initiating the progression of the autoimmune disease.

Recent studies indicate that DC act not only as stimulators but also as regulators of immune responses. For example, challenge with immature DC (either costimulation ^{low} or IL-12^{low}) loaded with self Ags, such as interphotoreceptor retinoid-binding protein (IRBP) peptide 161-180 or MBP peptide 68-86, induced a state of tolerance to the peptide-specific autoimmunity (Huang et al. 2000, Yang et al. 2000, Jiang et al. 2003). The tolerogenic properties of the DC are mediated by three distinct mechanisms: 1) Induction of anergy. It has been known that T cells encountering Ag presented by APC, which express low levels of co-stimulatory molecules, become anergic. IL-10-treated DC, characterized by reduced surface expression of MHC and costimulatory molecules and reduced expression of proinflammatory cytokines, were reported to induce Ag-specific T

cell anergy (Enk et al. 1993). In another study, IL-10-treated human DC, from peripheral blood, induced alloreactive $CD4^+$ and $CD8^+$ T cell anergy and an Ag-specific anergy in hemagglutinin or melanoma-specific $CD4^+$ and $CD8^+$ T cells (Steinbrink et al. 1997). 2) Elimination of autoreactive T cells. Using engineered Ab techniques, DNA coding for hen egg lysozyme (HEL) peptide 46–61 with spacing residues on both sides was fused to DNA encoding the C terminus of the heavy chain of anti-DEC205 Abs (Hawiger et al. 2001). After delivery of the hybrid Abs, which target specifically to DC in vivo, HEL peptide-specific $CD4^+$ T cells were deleted after a short proliferative burst. 3) Generation and/or expansion of regulatory T cells. "Semi-mature" , i.e. TNF-α –treated DC show increased expression of MHC class II and costimulatory molecules, but secrete low levels of pro-inflammatory cytokines such as IL-12 and TNF-α (Lutz and Schuler 2002). Immunization with TNF-α –treated DC expressing the MOG peptide ameliorates development of EAE induced by the same peptide, and the protection is probably mediated by IL-10-producing T cells (Menges et al. 2002). Our study confirmed this observation in EAT using Tg-pulsed, TNF-α-treated DC, and further demonstrated that the IL-10-producing T cells are $CD4^+CD25^+$ regulatory T cells, which mediate suppression in an Ag-specific, cell-to-cell contact - dependent manner (Verginis et al. 2005).

1.4.3 Cell death as a danger signal to break self tolerance

The term "danger signals" was first coined in 1994, referring to molecules or molecular structures that are released or produced by cells undergoing stress or abnormal cell death

(Gallucci and Matzinger 2001). During cell death, many proteins and nucleic acids are uniquely modified by various processes, including protease-mediated cleavage and (de)phosphorylation (Rodenburg et al. 2000), and new molecules, such as cytokines and chemokines, are synthesized (Gallucci and Matzinger 2001). These modifications uncover cryptic self-epitopes and/or create novel epitopes to which no tolerance exists, or recruit and activate resting APC provoking an autoimmune response.

There are two major types of cell death: apoptosis and necrosis. Apoptosis is a process of programmed cell suicide, which occurs during tissue remodeling. Early changes in apoptotic cells are nuclear blebbing, alteration in cell morphology, and eventually, fragmentation of the DNA with no loss of membrane integrity (Savill et al. 2002). Apoptotic cells are usually cleared by macrophages or DC via a non-inflammatory pathway. Immature DC phagocytose apoptotic cells peripherally and transfer the tissue-derived self peptides to naïve T cells upon reaching the draining lymph nodes, leading to induction of T cell anergy (Steinman et al. 2000). However, apoptotic cells in large numbers become sufficient to trigger DC maturation, even in the absence of exogenous "danger" signals (Ignatius et al. 2000, Rovere et al. 1998). In contrast, necrosis is a pathological process of cell disintegration caused by physical/chemical injury, or Ab/complement-mediated membrane damage (Savill et al. 2002). Necrotic cells release a range of endogenous adjuvant, such as genomic DNA (Ishii et al. 2001), heat shock proteins (Basu et al. 2000), high-mobility group B1 protein (Rovere-Querini et al. 2004) or uric acids (Shi et al. 2003). These components may work alone or together to stimulate

DC maturation in situ, turning tolerogenic DC to immunogenic DC. Initially, it was reported that only necrotic tumor cells, but not necrotic primary cells - T and B cells, monocyte and keratinocytes - were able to mature DC (Sauter et al. 2000). Yet, others showed that exposure to necrotic primary fibroblasts was also sufficient to induce DC functional maturation (Gallucci et al. 1999, Rovere-Querini et al. 2004). The contradictory results may be due to the different cell types used in each study, as well as the necrotizing procedures.

Immature DC are specialized in engulfment of dead cells in vivo and in vitro (Gaipl et al. 2003, Larsson et al. 2001). Ags derived from these cells are processed and presented onto MHC class II molecules for $CD4^+$ priming (Inaba et al. 1998), but they can also be cross-presented on MHC class I for $CD8^+$ CTL activation, a process defined as "cross presentation" (Larsson et al. 2001). Cross-priming is essential for the induction of $CD8^+$ T cell responses directed towards antigens not expressed in professional APC, such as dead cells or their derivatives. Studies with transgenic mice in which MHC class I expression is driven selectively in DC provided direct in vivo evidence that DC are sufficient to cross-present exogenous self-Ags and induce Ag-specific cell division of $CD8^+$ T cells (Kurts et al. 1997). Furthermore, the $CD8\alpha^+$, but not $CD8\alpha^-$, splenic DC subset was reported to mediate the cross presentation of cell-associated Ags in the context of MHC class I, after in vivo priming with ovalbumin-loaded ß2-microglobulin–deficient splenocytes (den Haan et al. 2000).

It has been proposed that the development of autoimmune disease is linked to abnormal cell death. This association was first noticed in MRL/lpr mice, which develop a lupus-like autoimmune syndrome, probably resulting from failure of proper clearance of autoreactive T cells caused by defects in Fas molecule (Watanabe-Fukunaga et al. 1992). A similar inheritable disorder, autoimmune lymphoproliferative syndrome (ALPS), was discovered in humans carrying mutations in genes involved in cell apoptosis, such as CD95 and caspase-10 (Straus et al. 1999, Wang et al. 1999). In the above examples, the systemic deficiency in cell death (i.e. too little cell death) appears to be a risk factor for autoimmunity, probably due to the inability of clearance of autoreactive lymphocytes. In NOD mice, however, it is proposed that an increase in β cell apoptosis at 12d of age may provoke the activation of islet-reactive T cells by augmenting release and presentation β cell-associated Ags by DC (Turley et al. 2003). In the case of iodide-induced EAT, it was found that the increased intrathyroidal iodine concentration promotes thyrocyte necrosis followed by mononuclear cell infiltration, suggesting thyrocyte necrosis may be an initial step in iodide-induced EAT in OS chickens (Bagchi et al. 1995). In the latter two studies, increased cell death in the target organ facilitates the development of organ-specific autoimmune disease.

Necrotic cells have been used as a source of Ag and proinflammatory stimuli in tumor therapy (Fields et al. 1998, Herr et al. 2000, Nestle et al. 1998a). Yet, only a few groups have attempted to induce autoimmune disease using DC exposed to necrotic primary cells. Myocarditis, a T cell-mediated organ-specific autoimmune disease, was

successfully induced in BALB/c mice by challenge with DC pulsed with apoptotic cardiomyocytes, followed by maturation with LPS and anti-CD40 (Eriksson et al. 2003). In systemic autoimmune disease, DC exposed to apoptotic thymocytes were reported to induce production of anti-nuclear and anti-dsDNA Abs with clinical features of systemic autoimmunity in autoimmune-prone (NZB X NZW) F1 mice, but not in normal mice (Bondanza et al. 2003, Bondanza et al. 2004, Georgiev et al. 2005). Even DC alone could induce A-Abs in recipient mice (Georgiev et al. 2005), but this remains unexplained and requires further investigation. In a recent paper, administration of DC exposed to necrotic splenocytes, but not apoptotic cells, induced strong anti-dsDNA antibodies and clinical manifestations of lupus in lupus-prone MRL/+ mice (Ma et al. 2005). In normal C57BL/6 mice, only anti-dsDNA Abs were induced and they were not accompanied by clinical symptoms. Furthermore, the A-Ab responses became significant only if adult (11 weeks of age) but not young mice (5 weeks of age) were used as recipients. This publication highlighted the difference of necrotic vs apoptotic cells in educating DC as immune stimulators, and pinpointed the important role of genetic background and age of the recipient.

1.5 THYMIC EXPRESSION OF AUTOANTIGENS

Immunological self-tolerance is a state in which the immune system does not destructively attack self-tissues. Lack or loss of self-tolerance is likely to result in autoimmune responses, eventually leading to the clinical onset of autoimmune disease. Induction and maintenance of immunological self-tolerance is mediated by multiple mechanisms operating both in the thymus (central tolerance) and the periphery (peripheral tolerance).

1.5.1 Mechanisms of central and peripheral tolerance

The thymus is considered as the primary site for the shaping of the T cell repertoire during intrathymic development (Sprent and Kishimoto 2001). T cell progenitors enter the thymus from the bone marrow, moving from the cortex to the medullary area during their maturation and selection. At the outer edge of the cortex, in the subcapsular region of the thymus, large immature $CD4^-CD8^-$ double-negative (DN,) thymocytes proliferate vigorously, differentiate into small $CD4^+CD8^+$ double positive (DP) thymocytes and move to a deeper area of the cortex. DP T lymphocytes recognizing self-peptide/MHC complexes on the cortical epithelial cells with low avidity are positively selected (Cosgrove et al. 1992). In contrast, developing T lymphocytes recognizing self-Ags on stromal or bone marrow-derived cells with high avidity are negatively selected via apoptosis in the thymic medulla (Sprent et al. 1996). Ag-specific T cell deletion or selection, therefore, requires the given self-Ag or its mimic to be either expressed in the thymus or enter the thymus via blood or lymph circulation. Recently, several studies have

demonstrated that naturally occurring $CD4^+CD25^+$ Treg cells are generated in the thymus through interaction with their specific ligands (Apostolou et al. 2002, Jordan et al. 2001, Grajewski et al. 2006). T cells that are positively selected in the thymus enter the circulation as mature single positive (either $CD4^+$ or $CD8^+$) lymphocytes.

The process of central tolerance (negative selection) does not preclude the presence of autoreactive T cells in the periphery. This is probably caused by either failure to encounter their ligands, such as tissue-specific Ags ("privileged Ag") in the thymus or, by receptor revision in the periphery (Lantelme et al. 2000, McMahan and Fink 2000). In peripheral lymphoid and/or nonlymphoid organs, several mutually nonexclusive mechanisms are proposed to secure tolerance of autoreactive T cells. First, the induction of anergy (Keir and Sharpe 2005). Activation of T cells requires not only the interaction between TcR and the MHC-peptide complex (first signal), but also costimulation from the APC (second signal). One of the most important costimulators is the B7 molecule on the APC and its ligand on the T cell designated CD28. At most of the time, the cells presenting the self Ags fail to provide the second signal, leading to T cell anergy instead of activation. Second, ignorance due to the spatial separation of T cells from their cognate Ags (Klein and Kyewski 2000). Some tissues hidden behind anatomical barriers keep T cells from reaching them ("privileged" site). Third, clonal elimination via apoptosis. Certain autoreactive T cell clones undergo extensive proliferation upon encounter with their ligands, then die by activation-induced cell death mediated by Fas ligand (Rocha and von Boehmer 1991, Webb et al. 1990). Fourth, the presence of Treg cells inhibit the

activity of potential effector cells, protecting the body from autoimmune attack (Hauben and Roncarolo 2005).

1.5.2 Intrathymic expression of tissue antigen

Many autoimmune diseases affect tissues that are anatomically sequestered from the immune system, such as brain, testis or eye. It was originally assumed that the major A-Ags in such diseases were expressed exclusively in the target organs, and self-tolerance was achieved only through peripheral tolerance (Mason and Powrie 1998, Webb et al. 1990). However, recent data has indicated that such A-Ags are expressed intrathymically at either the mRNA and/or protein level (Klein and Kyewski 2000). This phenomenon of promiscuous or ectopic gene expression has been considered to participate in central tolerance. The AIRE (autoimmune regulator) gene expressed predominantly in mTECs, has now been demonstrated to act as a master regulator of the intrathymic expression of many tissue-specific Ags (Gotter and Kyewski 2004).

A broad range of target A-Ags with tissue-restricted or mainly peripheral expression has been investigated for intrathymic expression. Among the pioneering studies, mRNA encoding insulin, an islet-specific target A-Ag in IDDM, was detected in mouse thymus (Jolicoeur et al. 1994) during fetal development and after birth. Similarly, myelin basic protein - a major A-Ag in EAE previously thought to be expressed exclusively in oligodendrocytes and Schwann cells, was detected at both the mRNA and protein level in normal SJL thymus (Fritz and Kalvakolanu 1995, Fritz and Zhao 1996). In 1997, it was

reported that intrathymic expression of two major uveitogenic retinal proteins, S-antigen and interphotoreceptor retinoid-binding protein (IRBP), varied significantly among different animal species and strains (Egwuagu et al. 1997). Other studies have revealed several peripheral antigens that are also expressed in human and/or mouse thymus, including proteolipid protein (PLP) – an A-Ag in EAE , glutamic acid decarboxylase (GAD) and the tyrosine phosphatase IA2 – A-Ags in IDDM, as well as TSHR, Tg and TPO –A-Ags in EAT (Egwuagu et al. 1997, Heath et al. 1998, Sospedra et al. 1998, Spitzweg et al. 1999a, Derbinski et al. 2001, Pugliese et al. 2001).

Initial studies showed that the mouse thymic medulla contains a small number of specialized cells expressing peripheral antigens such as insulin (Smith et al. 1997). Later studies by Throsby *et al.* (Throsby et al. 1998) and Pugliese *et al.* (Pugliese et al. 2001) demonstrated that thymic cells expressing islet cell antigens belonged to the DC and macrophage lineage in mice and humans. In contrast, promiscuous gene expression was also detected in thymocytes (Mor et al. 1998) and thymic epithelial cells (Derbinski et al. 2001). In a recent extensive and elegant study, Kyewski *et al.* have demonstrated that nearly all genes encoding autoimmune target antigens are expressed in mTEC (Kyewski et al. 2002). Among them, a few are simultaneously detected in BM-derived DC or macrophages, or cortical TEC. Negative selection in the thymus is driven most efficiently by bone marrow-derived APC, such as DC and macrophages. In addition, both thymocytes themselves and thymic epithelial cells have the ability to cause the deletion of self-reactive cells (Sprent et al. 1996). Nevertheless, it remains inconclusive as to what

extent, and how, each cell subpopulation contributes to the deletion of autoreactive T cells.

1.5.3 "Leakage" of central tolerance

The presence of potentially autoreactive T cells in the normal thymic repertoire is a well-documented observation (Klein and Kyewski 2000). Several mechanisms that control the quantity and quality of the intrathymic gene expression may contribute to the incompleteness of central tolerance. The first mechanism operated at the level of transcription in the thymus. In the EAU model, it was shown for the first time that constitutive expression of ocular S-antigens and IRBP in the thymus correlated with resistance to EAU among different species and strains (Egwuagu et al. 1997). Moreover, IRBP-knockout mice had greatly elevated response to IRBP, an altered recognition of IRBP epitopes, as well as exacerbated disease, after deliberate immunization (Avichezer et al. 2003). Similarly, when two different sets of MHC congeneic strains of mice were compared, increased thymic expression of MBP was reported in mice that are more resistant to EAE (Liu et al. 2001a). Furthermore, the level of insulin mRNA expression in the human thymus is associated with allelic variation at the IDDM2 susceptible locus, and the transcription activity in the thymus was found to be about 2-3 fold higher for insulin transcripts encoded by IDDM2 alleles that were clinically associated with resistance to the development of diabetes (Pugliese et al. 1997). Lastly, it was proposed that reduced thymic expression of islet antigens, such insulin (Brimnes et al. 2002) and ICA69 (Mathews et al. 2003), may predispose NOD mice to spontaneous development of IDDM.

The second mechanism operates when the A-Ag expressed in the thymus is different from that in the specific organ. For example, in EAE, the predominant form of PLP expressed in thymic epithelial cells is DM, a splice variant that lacks residues 116-150 of the full-length PLP in the central nervous system (Klein et al. 2000). T cells specific for residues that are absent in the thymus do not undergo negative selection and can "escape" from the thymus to the periphery, i.e. the $PLP_{139-151}$ – reactive T cells. Reexpression of $PLP_{139-151}$ in the embryonic thymus results in a significant reduction of $PLP_{139-151}$-reactive precursors in naïve SJL mice (Anderson et al. 2000). Similarly, IA-2 transcripts in thymus and spleen are present in an alternatively spliced form lacking exon 13, whereas pancreatic islets express full-length mRNA and two alternatively spliced transcripts (Diez et al. 2001). The differential splicing was proposed as a regulatory mechanism to play a permissive role in the development of autoimmune responses to IA2.

The third mechanism operates when thymically expressed A-Ags do not mediate T cell deletion due to inefficient antigen presentation by MHC alleles or low affinity recognition by TcR. This mechanism has been proposed to explain the escape of MBP 1-11-reactive T cells in mice bearing the $H\text{-}2^u$ haplotype. MBP 1-11 is expressed in mouse thymus, but binds only weakly to $I\text{-}A^u$ and form unstable peptide-MHC complexes (Anderson and Kuchroo 2003). Such peptide-MHC complexes are inefficient to mediate thymic deletion, leading to the escape of T cells recognizing the MBP 1-11 epitope; but they are sufficient to induce T cell activation in the periphery (Harrington et al. 1998). MHC instability and

poor peptide binding may also account for the susceptibility of NOD to autoimmunity, since the I-A^{g7} alleles have been shown to be structurally unstable and to bind to diabetes-related self-peptides poorly (Carrasco-Marin et al. 1996).

All of the mechanisms illustrated above refer to the negative selection mediated by self antigens expressed in the thymus. Yet, it is not known whether such promiscuous expression is also involved in positive selection or generation of regulatory cells in the thymus. On the other hand, lack of thymic expression may not always preclude thymic deletion of self-reactive T cells. First, some self antigens can enter the thymus via circulation, either in soluble form, e.g. Tg (Pugliese 2004) or are expressed in hematopoietic cells, e.g. Tg and PLP (Bugalho et al. 2001, Klein et al. 2000). Second, the expression of genes encoding self-molecules is not limited to the thymus but also take place in peripheral lymphoid organs, e.g. insulin, where it may contribute to the peripheral tolerance (Pugliese 2004).

1.6 HYPOTHESIS AND OBJECTIVES OF THE STUDY

Hypothesis 1 (Chapter 3&4):

Normal Tg may harbour several pathogenic T -cell epitopes that contain monoiodotyrosyl(s) outside the hormonogenic sites.

Objectives:

1.1 To identify murine Tg peptides that contain A^k-binding motif flanked by Tyr residues(s).

1.2 To examine the effects of iodotyrosyl formation on the immunopathogenicity of the above peptides by determining the immunopathogenic profiles of both non-iodinated and iodinated peptides in CBA/J mice.

1.3 To explore the mechanisms by which iodination of Tyr residue(s) may impart immunogenicity to a Tg peptide.

1.4 To identify Tg peptides encompass (sub)dominant T cell epitope among the immunogenic peptides identified in the above study.

Hypothesis 2 (Chapter 5):

Administration of iodide to SJL and CBA/J mice might promote Tg iodination in vivo, and sensitization of autoreactive T cells of the hosts to pathogenic Tg determinants.

Objectives:

2.1 To determine whether administration of iodide (0.05% NaI in drinking water) to SJL and CBA/J mice will facilitate the development of EAT.

2.2 To explore the effects of iodide administration on iodide organification and thyroid hormone secretion, by examining the iodine content in Tg purified from the experimental and control animals, and the serum levels of thyroid hormones (i.e. total T4 and TSH).

2.3 To examine whether iodide administration exerts differential effects on SJL vs CBA/J mice. If there is a difference, to explore the mechanisms by which this phenomenon occurs.

Hypothesis 3 (Chapter 6):

Exposure to necrotic thyrocytes may trigger DC maturation. This enables the immunogenic presentation of thyroid antigen(s) – such as Tg – on the DC surface, leading to development of EAT in CBA/J mice.

Objectives:

3.1 To establish primary culture of mouse thyrocyte and bone marrow-derived DC.

3.2 To determine whether necrotic (induced by 4 times of freezing/thawing cycles) – but not viable - thyrocytes can mediate both phenotypic and functional maturation of DC.

3.3 To examine whether DC that have ingested syngeneic necrotic thyrocytes can elicit EAT or thyroid antigen-specific responses, following their adoptive transfer in CBA/J hosts.

Hypothesis 4 (Chapter 7):

The apparently contrasting results of RT-PCR detection of intrathymic Tg expression, the large size of the Tg molecule (2748 aa), and the expression of A-Ag isoforms in the thymus in other animal models, prompted us to hypothesize that Tg transcripts may be present in differential isoforms in thymus vs thyroid in CBA/J mice.

Objectives:

4.1 To determine whether Tg transcripts are present in differential isoforms in mouse thymus vs thyroid, via RT-PCR using primers amplifying 12 Tg pathogenic peptides that scatter through the whole Tg gene.

4.2 To examine the presence of Tg transcripts in tissues other than thyroid and thymus, such as liver, kidney and muscle.

4.3 To determine whether the expression profile of Tg transcripts encoding the 12 Tg pathogenic peptides changes over time after birth.

1.7 CO-AUTHORSHIP STATEMENT

Hong Jiang, a current M.Sc. student in the laboratory, participated in the study of Chapter 4 by performing some experiments as indicated on page 132. Dr. Panayotis Verginis provided help with the experimental design of the studies conducted in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Female CBA/J (H-2^k), C3H/HeJ (H-2^k), SJL/J (H-2^s) mice and breeding CBA/J pairs were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). For some experiments, mice were placed on drinking water containing 0.05 % sodium iodide (NaI) over a variable time period. All experimental procedures were reviewed and approved by the Animal Care Committee at Memorial University of Newfoundland.

2.2 ANTIGENS AND ANTIBODIES

2.2.1 Tg and its iodination

Tg was extracted from Tg thyroids of outbred ICR mice as previously described (Chronopoulou and Carayanniotis 1992). Briefly, frozen thyroid glands (Bioproducts for Science, Indianapolis, IN) were homogenized in phosphate buffer in the presence of protease inhibitors: Leupeptin 10^{-6} mol/L, Pepstatin A 10^{-5} mol/L, PMSF 10^{-3} mol/L, and the supernatant was collected after 3 X centrifugation at 14,000 xg. Tg was obtained from the supernatant by gel filtration using Sepharose CL-4B (Pharmacia, Baie d'Urfé, Quebec, Canada). The fractions of peak II (**Fig 2.1**) were pooled, concentrated by using ultra-filtration cells (Amicon, Danvers, MA) and filter-sterilized to a final concentration of 2-3 mg/ml, and stored at –20 °C until use. In some cases, Tg was lyophilized.

Tg iodination was performed in 12 x 75 mm glass tubes coated with IODOGEN (Pierce, Rockford, Illinois). As previously described (Dai et al. 2002), 100 μl of 1 mg/ml Tg was incubated with 0.25 mM/10 μl NaI in each tube coated with 10 μg of IODOGEN at room temperature for 0-20 min. Free iodine was removed by dialysis in PBS. The iodinated Tg (HI-Tg) was concentrated and filter-sterilized. The iodine content in Tg samples was determined by a modified non-incinerative method based on the catalytic activity of iodine in the ceric (Ce)-arsenite (As) reaction (Saboori et al. 1993). The reduction of Ce(IV) to Ce(III) by As(III) leads to a decoloration of yellow ceric ion to colorless cerious ion, a process which can be followed spectrophotometrically. Construction of the standard curve was performed using known concentration of T4 dissolved in 99 vol of absolute methanol and 1 vol of 30% ammonium hydroxide. The iodine content in normal Tg and HI-Tg was extrapolated by the standard curve. All the HI-Tg used in this study contains iodine ranging from 60-75 atoms per Tg monomer.

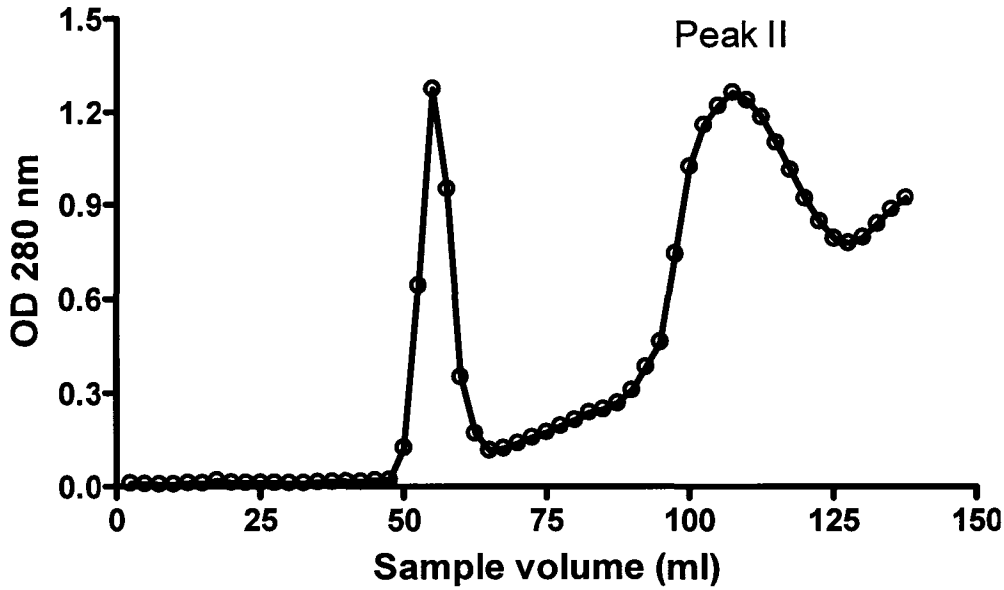

Figure 2. 1 Purification of mouse Tg.

One hundred thyroid glands from ICR mice were homogenized in 2.5 ml ice-cold PBS in the presence of protein inhibitors as described in Materials and Methods. The supernatant was loaded on a Sepharose column after 3 times centrifugation at 14,000 xg. Tg was eluted with PBS and collected in 2.5 ml per tube for subsequent OD measurement. Fractions from tubes 32 – 47 (80 to117 ml of the elution) within peak II were pooled, dialyzed and concentrated in double distilled H_2O. The Tg samples were further filtered, lyophilized and stored at -20 °C.

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

A computerized algorithm was developed by Altuvia *et al.* to identify potential A^k-binding a.a. sequence in peptides or proteins (Altuvia et al. 1994). This approach has been used in our laboratory to search for A^k-restricted Tg pathogenic peptides (Verginis et al. 2002). In this study, the complete murine Tg sequence (Kim et al. 1998) was scanned for the presence of peptides containing either of the two I-A^k-binding motifs, a heptamer motif A and a pentamer motif B, flanked by one or more Tyr residues within 5 aa positions from the N- or C-terminus of the motif. The website "Pole Bio-Informatique Lyonnais: Network Protein Sequence Analysis" (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_pattinprot.html) was used for Tg scanning in the study. Peptides containing Pro residues within the motif or between Tyr residues and the motif were excluded because of concerns that Pro may drastically affect the secondary structure.

All candidate peptides and their iodinated analogs (Table 3.1) were synthesized and purified by Dalton Chemical Laboratories Inc. (Toronto, Canada). F-moc-3-iodo-tyrosine was used for synthesis of iodinated peptide analogs. Iodine content in the noniodinated and iodinated analogs was confirmed by method described in Section 2.2.1. Other mouse Tg peptides, such as p2494 and p2596, used in the study were previously synthesized at the Alberta Peptide Institute (Edmonton, Alberta, Canada). All peptides were blocked with an acetyl group at the N-terminal end and an amide group at the C-terminal end, and the –SH group of the internal Cys residues was blocked by acetamide. Mass spectrometry

and HPLC analysis were performed on each peptide to verify composition and confirm >80% purity.

2.3 CELL LINES AND TISSUE CULTURE

2.3.1 Cell lines

MAb-secreting B cell hybridoma clones were purchased from ATCC, including: HB 32 (14-4-4S, mouse IgG2α against I-E^k), HB 65 (H16-L10-4R5, mouse IgG2a against nucleoprotein of influenza type A virus), TIB 92 (10-3.6.2, IgG2α mAb against I-A^k). The B cell hybridomas were cultured in a spinner flask, which contained 500 ml medium on the 1st day, with 500 ml of medium and 1000 ml of medium added on the 2nd and 3rd day, respectively. After a 5-day culture, mAbs were purified from culture supernatants by affinity chromatography on protein G-Sepharose 4 Fast Flow columns (Pharmacia, Baie d'Urfé, Quebec, Canada).

The T-cell hybridoma 4A12 (I-A^k-restricted, p2498-specific) and 8F9.27 (E^k-restricted, p2494-specific) were generated and characterized previously (Carayanniotis et al. 1994, Rao et al. 1994). The T cell hybridomas (4A6, 10C1 and 1H7), specific for Tg peptides I-p117, I-p304, I-p1931, were generated following a modified method of Perkins *et al.* (Perkins et al. 1991). Briefly, mice were primed with 100nm of the specific Tg peptide in CFA, 9 days later draining LNC were removed and cultured in the presence of 20 μM of respective peptide for 3 days. These cells were then fused at 1:2 ratio with BW5147$\alpha^-\beta^-$ lymphoma variant (White et al. 1989) - a kind gift from Dr. P. Marrack (National Jewish

Centre, Denver, CO, USA), using polyethylene glycol (Boehringer Mannheim, Indianapolis, IN). Fusion products were selected in medium containing 1X oxanthine-Aminopterin-Thymidine, and gradually through medium containing 1X oxanthine-Thymidine to normal medium. The specificity and sensitivity of the hybridoma was tested by activation assay as described below. The peptide - specific hybridomas were cloned by limiting dilution at 0.3 cell/well using 1% syngeneic red blood cells (RBC) as feeder cells.

The APC cell line TA3 (I-$A^{k/d}$, $E^{k/d}$), produced by fusion of B cells from CAF1 mice with M12.4.1 BALB/c B lymphoma (Allen et al. 1985), was a kind gift from L. Glimcher (Harvard medical school), and courteously provided by Dr. T. Watts (University of Toronto). The other two APC cell lines, LS 102.9 (I-$A^{d/s}$, E^{d}) and LK 35.2 (I-$A^{k/d}$, $E^{k/d}$) are B cell hybridomas obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) (Kappler et al. 1982). The CTLL-2 cell line (ATCC) (Gillis and Smith 1977) is was grown in DMEM supplemented with 10% IL-2-containing supernatants, obtained from rat splenic cells activated with 5 μg/ml of concanavalin-A. The XG6 cell line transfected with murine GM-CSF gene and spontaneously secreting GM-CSF (Zal et al. 1994) was a kind gift from Dr. B. Stockinger (National Institute for Medical Research, London, United Kingdom).

All tissue culture media including Ham's F12, RPMI-1640 and Dulbecco's Modified Eagle's Medium (DMEM) were supplemented with 10% or 5% heat-inactivated fetal

bovine serum (FBS) (Cansera, ON, Canada), 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco). RPMI-1640 and DMEM were also supplemented with 20mM HEPES (Gibco) and 5 x 10^{-5} M 2-ME (Sigma).

2.3.2 Primary thyrocyte culture

Fresh mouse thyrocytes were isolated from CBA/J mouse thyroid according to an established protocol (Caturegli et al. 2003, Jeker et al. 1999). Briefly, two thyroid lobes were fragmented and digested for 30 min in 250 μl of digestion medium, which consists of 112 units/ml of Type I collagenase (Sigma, St. Louis, MO) and 1.2 units/ml of dispase (Roche Diagnostics Corp, Indianapolis, IN) in EMEM. After digestion, thyroid follicles were pelleted, resuspended and cultured in a Petri dish or 6-well plate in F-12 medium supplemented with Nu-Serum IV (BD Biosciences), 10 ng/ml somatostatin (Sigma), and 2 ng/ml glycyl-L-histidyl-L-lysine acetate (Sigma).

To confirm the thyrocyte origin of these cells, digested thyroid sections were grown in 8-well chamber slides (Nalge Nunc Internationa, Naperville, IL, USA) for one week. The cells were fixed in acetone at -20°C for 5 min, and incubated in the presence of rabbit polyclonal Abs specific for human Tg which crossreact to murine Tg (DAKO, Carpinteria, CA, USA). Following 1 hr incubation, the cells were washed, and a secondary Ab, FITC-conjugated goat-anti-rabbit Abs (Sigma), were added and incubated for 30 min. Finally, slides were mounted in 50 % glycerol in PBS and sealed for

immunofluoresece imaging under an Olympus Fluoview 300 Laser scanning confocal microscope.

One week after culture, thyrocyte necrosis was conducted by four freeze-thaw cycles from -80°C to room temperature (Fields et al. 1998, Heimberger et al. 2000, Herr et al. 2000, Nestle et al. 1998b, Vegh and Mazumder 2003). Disruption of the cell membrane was measured by trypan blue staining.

2.3.3 Generation of bone marrow-derived dendritic cells

Bone marrow-derived DC were generated according to the protocol developed by Inaba (Inaba et al. 1992) and modified by Lutz (Lutz et al. 1999). As described in a previous study (Verginis et al. 2005), bone marrow leukocytes were collected from femurs and tibias of female CBA mice, and RBCs were lysed by NH_4Cl. The leukocytes were then seeded at 2 X 10^6 cells per 100 mm^2 petri dish in 10 ml complete RPMI 1640 medium supplemented with 10% supernatant from the murine- GM-CSF-secreting X63Ag8 cell line (Zal et al. 1994). On day 3, another 10 ml of media containing GM-CSF was added to each plate. At days 6 and 8, half of the culture supernatant was collected, centrifuged, then the cell pellet was resuspended in 10 ml fresh medium containing 10 % X63Ag8 supernatant and the cells were returned onto the original plate. On day 10, non-adherent cells were gently dislodged and collected for future experiments.

2.4 T CELL ACTIVATION/INHIBITION ASSAY

2.4.1 T-cell hybridoma activation assay

Activation of Tg peptide-specific T-cell hybridomas was performed in a 96- well flat bottom plate. Briefly, 10^5 hybridoma T cells and an equal number of APC were cultured for 24 h with or without the respective antigen in a 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 more 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 (PerkinElmer, Life and analytical sciences, Boston, MA) was added to each well in 25 µl of complete medium. The cells were harvested 6 h later 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).

2.4.2 T-cell hybridoma blocking assay

MHC restriction of TcRs expressed on the T cell hybridoma clones was determined in a blocking assay. Briefly, in a 96-well plate, 10^5 T cell hybridomas (4A6, 10C1, 1H7) were cocultured with 10^5 LK35.2 cells in the presence of a constant amount of their respective ligand and serially diluted anti-A^k (TIB92), anti-E^k (HB32) or control Abs (HB65). Following 24 h incubation, 100 µl of supernatant was removed and stored at –20°C for assessment of IL-2 release by CTLL-2 assay. The data are expressed as follows: % inhibition = [1 - (cpm in the presence of mAb)/(cpm in the absence of mAb)] x 100.

2.4.3 T-cell hybridoma competitive inhibition assay

A competitive inhibition assay was used to determine the peptide binding to A^k. In this assay, 10^5 A^k-restricted T-cell hybridomas (4A12, p2494-specific) were cocultured with 10^5 LK35.2 cells in flat-bottom wells of 96-well plates in presence of a constant amount of the respective ligand (1 μM). Serial dilutions of the inhibitor peptides (starting from 100 μM) were added in triplicates, in a total volume of 200 μl per well. Following 24 h incubation, 100 μl of supernatant was removed and stored at –20°C for assessment of IL-2 release, as measured by the proliferation of CTLL-2 line.

2.5 T CELL PROLIFERATION ASSAY

2.5.1 LNC proliferation assay

Experimental mice were challenged s.c. with 100 nmol of peptide, or 100 μg of Tg, in 100 μl of CFA). Nine days later, inguinal, axillary, and branchial ln were collected aseptically and single cell suspensions were prepared in complete DMEM medium supplemented with 10% fetal bovine serum (FBS) (Cansera, Ontario, CA), 20 mM HEPES buffer, 2 mM 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). After centrifugation and washing, 4×10^5 cells/well per 200 μl were cultured in 96-well plates for 4 days in the presence of titrated amounts of the appropriate antigens. During the last 18 hrs, 1 μCi of [3H]-thymidine was added to each well in 25 μl of culture medium. Cells were

harvested using the Harvester 96 ® Mach III M (Tomtec, Hamden, Ct, USA) and incorporated radioactivity was measured using the TopCount NXTTM Microplate Scintillation & Luminescence Counter (Canberra Packard Canada, Mississauga, Ontario, Canada). Stimulation index (S.I.) is defined as (cpm in the presence of peptide/cpm in the absence of peptide).

2.5.2 Splenic $CD4^+$ T cell proliferation assay

Mice were challenged s.c. with peptide or Tg in CFA and boosted with respective antigen in IFA. Two weeks after boosting, spleens were collected for detection of antigen-specific T cells. Briefly, a single cell suspension is made in complete DMEM medium, and RBCs lysed by NH_4CL. Splenic $CD4^+$ T cells were isolated using MACs separation according to the manufacturer's instruction (Miltenyi Biotec Inc., Auburn, CA). Briefly, splenocytes were incubated with a cocktail of biotin-conjugated mAbs against CD8α, CD11b, CD45R, DX5 and Ter-119, then anti-biotin microbeads were added to the mixer. The magnetically labelled non-$CD4^+$ cells were depleted by retaining them in a MACS column in the magnetic field of a MACS Separator. $CD4^+$ splenocytes were cultured at 2×10^5 cells/well with 2×10^5 cells/well of mitomycin-treated splenocytes or BM-DC for 3 days in the presence of titrated amounts of the appropriate antigens in a total of 200 μl microculture in 96-well plates. During the last 18 hrs, 1 μCi of [^{3}H]-thymidine was added to each well in 25 μl of culture medium. Cells were harvested and S.I. was calculated as Section 2.5.1.

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.6.1 Detection of IgG Ab

The presence of Tg or peptide- specific IgG antibodies (Ab) in primed sera was determined by ELISA. Briefly, 96-well plates were coated with 1 μg of Tg or 0.2 μg of peptide in 100 μl of carbonate buffer (pH 9.6), and incubated overnight at 4°C. Wells were then blocked with PBS containing 1% BSA for 1 hr at room temperature. Plates were washed 3 times in PBS/Tween and incubated at 4 °C overnight with 1:2 serially diluted sera with a starting dilution of 1:30. The binding of Abs in the sera to the coated antigen was detected with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Sigma), and light absorption of p-nitrophenylate product (Sigma) at 405 nm was determined using a Vmax plate reader (Molecular Devices). The comparison of OD values of Ab ELISA was performed using t test in GraphPad Prism 4.0 software.

2.6.2 Detection of cytokines in culture supernatants

For activated LNC or splenic cells, cytokine production was determined in culture supernatants harvested after 48-h stimulation with optimal dose (5-20 μM) of antigen. Detection of IL-2, IL-4, IL-10, and IFN-γ was performed by sandwich ELISA using OptEIA™ ELISA kits (BD PharMingen, San Diego, CA), following the manufacturer's protocol. Similarly, culture supernatants were collected from DC treated with various stimuli to determine the IL-12 content. According to the manufacturer's protocol, 96-well polyvinyl chloride microwell plates were coated with capture Abs and blocked with

FBS. Cytokine standards and samples were then applied in duplicate (100 µl/well) and the plates were incubated at room temperature for 2 hours. The mixture of biotinylated detection antibodies and avidin-conjugated horseradish peroxidase were added, followed by the addition of substrate solution – Tetramethylbenzidine and hydrogen peroxide (BD Pharmingen). After incubation at room temperature for 30 min, the reaction was stopped by 2 N H_2SO_4. Absorbance at 450 nm was measured by using a Vmax plate reader (Molecular Devices, Sunnydale, CA, USA).

2.6.3 FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Expression of TcR-associated surface markers on T cell hybridomas was determined by FACS, using FITC-conjugated hamster anti-CD3ε (145-2C11) (BD Biosciences, San Jose, CA, USA) as well as PE-conjugated rat anti-CD4 (CT-CD4) or anti-CD8 (CT-CD8a) (Caltag Lab, Burlingame, CA, USA). Similarly, flow cytometry was used to determine the expression of MHC class II and costimulatory molecules on bone marrow-derived DC using the following antibodies: PE-anti-CD11c (HL3), FITC-anti-I-A^k (10-3.6), FITC-anti-CD80 (16-10A1), FITC-anti-CD86 (GL1), FITC-anti-CD40 (3/23), and FITC-anti-CD8α (53-6.7) (all from BD Biosciences).

The experiments were performed as follows: cells were harvested, washed and resuspended in FACS buffer (PBS/1% BSA/0.1 % NaN_3) to a concentration of 10^7 cells/ml. 10^6 cells per 100 µl were incubated with 1 µg of PE-labeled anti-CD4 Abs (CALTAG laboratories, Burlingame, CA, USA) and FITC-labeled anti-CD3 (BD

Pharmingen), or similarly labeled isotype control Abs (CALTAG laboratories). Following 30 min incubation on ice, the cells were washed 3 times with FACS buffer and suspended in 500 µl of 1x PBS buffer. The fluorescence of 10^4 cells was measured using BD FACS Calibur bench top flow cytometer and data analysis was performed by "Cell Quest Pro" (BD Biosciences).

2.7 EAT INDUCTION

2.7.1 Immunization with Tg peptides in CFA

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 emulsion of PBS:CFA. Three weeks later, they were boosted s.c. with 50 nmol of Tg peptide in IFA. Five weeks after the initial challenge, mice were bled to obtain sera for ELISA assays, and the thyroids were removed and fixed in 10% buffered formalin for histological examination.

2.7.2 Adoptive transfer of Tg peptide-primed LNC

Induction of EAT by adoptive transfer of Tg peptide-primed LNC was 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 LNC (4 X10^6/ml) were cultured in the presence of the immunizing peptide (20 µM) for 3 days. The cells were harvested and washed completely 3x with PBS and resuspended in PBS at 10^8 cells/ml. Syngeneic

recipient mice were intraperitoneally (i.p.) injected with 200 µl PBS containing $2X10^7$ activated LNC. Fourteen days after transfer, mice were sacrificed and their thyroid glands were collected in 10% buffered formalin for histological examination.

2.7.3 Vaccination with DC exposed to Tg or necrotic thyrocytes

Freshly isolated DC were gently dislodged and cultured overnight at 2×10^7 cells per 10 ml in the presence of 200 µg/ml Tg, or control antigen (200 µg/ml ovalbumin, OVA), followed by treatment with lipopolysaccharide (LPS, 1 µg/ml, 6 hr). Similar numbers of DC were also co-cultured with approximately 2×10^6 necrotic or viable thyrocytes for 24 hr. DC incubated with soluble antigens or thyrocytes were washed twice with PBS and 2×10^6 cells per 0.2 ml PBS were injected i.p. into syngeneic recipient mice. Two weeks later, the mice were boosted with same number of similarly treated DC. Thyroid glands were removed 2 weeks after boosting, and were sectioned and stained with hematoxylin and eosin (H & E) for histological examination.

2.7.4 Thyroid histology

Thyroid glands were removed and fixed in 10% buffered formalin. Fixed thyroid glands were embedded in methacrylate and sectioned serially (approximately 21 sections at 3.0 µm intervals were obtained/gland). They were fixed to glass slides and stained with hematoxylin and eosin and 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. The highest infiltration score observed per gland was assigned to each mouse. Statistical comparison of EAT scores between various groups was performed using Wilcoxon signed rank test in GraphPad Prism 4.0 software.

2.8 IODIDE-INDUCED HYPOTHYROIDISM

2.8.1 Animal treatment with dietary iodine

Female CBA/J and SJL mice at age of 4-6 weeks were purchased from the Jackson Laboratories (Bar Harbor, ME), and divided into two groups: control group (normal tap water), and NaI-treated group (0.05% NaI in drinking water for 10-12 weeks). All experiments were conducted in accordance with standard policies of the animal care committee at Memorial University of Newfoundland.

2.8.2 Determination of TSH and total T4 levels using radioimmunoassay (RIA)

Serum TSH level was determined by Dr. A.F. Parlow (Harbor-UCLA Med Ctr, Torrance, CA, USA). The DYNOtest T4 kit was purchased BRAHMS Diagnostica GmbH (Berlin, Germany) for quantitative determination of total thyroxine (T4). As described in the manufacturer's guide, reference T4 standards and samples were added to tubes coated with anti-T4 antiserum, T4 labeled with ^{125}I was subsequently added to the tube as tracer to compete for antibody binding. After a 2-h incubation at room temperature, the coated

tubes were dried on blotting paper and radioactivity was measured in Wallac 1277 GammaMaster (Perkin Elmer life sciences, Boston, MA, USA). The total T4 value in each sample was extrapolated from the T4 standard curve and expressed in μg/dl. Statistical comparisons of serum thyroid hormone levels were performed using t test (GraphPad Prism 4.0 software).

2.9 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

2.9.1 Total RNA isolation from tissues

Total RNA of freshly obtained thyroids from CBA/J or SJL/J mice was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, tissues were homogenized using disposable RNase-free homogenizers in 0.8 ml (1-10 mg of tissue) or 1 ml (> 10 mg of tissue) of TRIzol reagent. Following sample lysis, 200 μl of chloroform (Sigma Chemicals) was added, and the phase separation was achieved by centrifugation. The upper aqueous phase (~ 600 μl) was transferred in a new RNase-free tube and RNA was recovered by precipitation with isopropyl alcohol (500 μl). For mRNA isolation from small tissues, 5-10 μg of glycogen (Invitrogen) were added as carrier to the aqueous phase prior to isopropyl alcohol. The isolated RNA was washed with ethanol, air-dried and dissolved in RNase-free water (Sigma Chemicals). The RNA yield was measured using a Beckman Du®64 spectrophotometer (Fullerton, CA, USA) at the optical density of 260 and 280 nm.

2.9.2 cDNA synthesis

First-strand cDNA synthesis was carried out with total RNA using a cDNA synthesis kit (Amersham Bioscience, Buckinghamshire, UK). Briefly, 5 μg of total RNA was heated at 65°C for 10 min to remove any RNA secondary structure, and then chilled on ice. The RNA was added to a mixture containing 11 μl first-strand bulk mix and 1μl dithiothreitol (DTT) solution and 0.2 μg (1μl) NotI-d$(T)_{18}$ (all provided in the kit). The first-strand bulk mix contains $FPLC_{Pure}^{TM}$ murine reverse transcriptase, which catalyze the reaction. The mixture was incubated for 1 hour at 37°C and terminated by heating at 75 °C for 10 min. The cDNA was stored at -20°C.

2.9.3 Polymerase chain reaction (PCR)

PCR was performed in 50-μl reactions consisting of 2 μl cDNA, 2 μl 10 × PCR buffer (Life Technologies, Invitrogen, Paisley, UK), 2 μl of 50 mM $MgCL_2$ (Life Technologies), 0.4 μl of 25 mM dNTPs; Gibco), 1.5 μl forward primer (10 pmol/μl), 1.5 μl reverse primer (10 pmol/μl), 0.2 μl PlatinumTaq DNA polymerase (Life Technologies) and 37.4 μl nuclease-free water. The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 25-35 cycles of denaturation at 94°C for 1 min, annealing at 55-65°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The annealing temperature in each PCR reaction was adjusted to at leat 5°C below the lowest melting temperature of primers to be used. When using semiquantitative RT-PCR to assess the expression levels of multiple transcripts from the same sample, PCR cycles

(25 cycles for GAPDH and Tg, and 30 cycles for other genes) were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification. These reactions were run on a Perkin-Elmer thermocycler (Cetus, Norwalk, CT). All primers for genes encoding Tg pathogenic peptides used in chapter 7 or genes involved in iodine organification used in Chapter 5 were and were synthesized by Integrated DNA Technologies, Inc (Coralville, IA, USA). In some primers, artificial start and stop codons (ATG and GAT) as well as unique *KpnI* and *XbaI* restriction sites were introduced in the forward and reverse primers, respectively. PCR products were separated on agarose gels and were visualized by staining with ethidium bromide. The relative intensity of the pCR products was quantified by ChemiImager 4000 software (Alpha Innotech, Corp., San Leandro, CA). Relative expression was calculated as the ratio of the relative optical density of the Tg fragment to that of the β-actin or GAPDH in the same sample and under similar conditions of amplification.

CHAPTER 3

IODINATION OF TYROSYLS IN THYROGLOBULIN GENERATES NEOANTIGENIC DETERMINANTS THAT CAUSE THYROIDITIS

(This work was published in *J. Immunol. 2006 May; 115 (1): 85-9.* This is a modified version)

Haiyan S. Li and George Carayanniotis

Divisions of Endocrinology and Basic Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

3.1 ABSTRACT

Thyroglobulin (Tg) is unique in its ability to incorporate and store available iodine in the form of iodotyrosyl residues. Iodination of Tg has been known to increase its immunopathogenicity in experimental animals, presumably through the formation of iodine-containing neoantigenic determinants which can elicit an autoimmune response, but defined pathogenic Tg peptides carrying iodotyrosyls have not yet been identified. We report here that a systematic, algorithm-based search of mouse Tg has delineated three iodotyrosyl-containing peptides that activate autoreactive T cells and cause experimental autoimmune thyroiditis (EAT) in normal CBA/J mice. These peptides, (aa. 117-132, 304-318, and 1931-1945), were not immunogenic in their native form and iodination of tyrosyls facilitated either peptide binding to MHC or T-cell recognition of the peptide. These results demonstrate that iodotyrosyl formation in normal Tg confers pathogenic potential to certain peptides which may otherwise remain innocuous and undetectable by conventional mapping methods.

3.2 INTRODUCTION

Among the known A-Ags, Tg is unique in its ability to incorporate and store available iodine in the form of iodotyrosyl residues (Dunn and Dunn 2000). This process facilitates thyroid hormone - i.e. T4 and T3 -formation through intramolecular coupling of specific iodotyrosyls, but it also has immunological consequences: enhanced iodination of Tg has been known to increase its immunogenicity at the T- and B-cell level as well as its pathogenicity in experimental animals (Champion et al. 1987, Dai et al. 2002, Saboori et al. 1998, Sundick et al. 1987). The mechanisms underlying these observations remain mostly unknown, but progress with T-cell epitope mapping in Tg has recently shed light on some of the processes involved.

So far, thirteen Tg peptides encompassing T-cell epitopes that cause EAT have been identified and none of them has been classified as dominant (Carayanniotis 2003). Eleven of these peptides elicit EAT of considerable severity, but do not contain iodine, clearly highlighting that iodine atoms per se are not necessary for thyroiditogenicity. However, processing of HI-Tg, containing 55-70 atoms per monomeric subunit, facilitates generation of one of these pathogenic peptides (aa 2494-2510) (Dai et al. 2002), in agreement with earlier studies showing that the proteolytic degradation of Tg is affected by its iodine content (Dunn et al. 1983, Fouchier et al. 1983, Lamas and Ingbar 1978). On the other hand, experiments with Tg peptides containing hormonogenic sites have shown that iodine atoms can be an integral part of ligands recognized by thyroid-infiltrating T cells. The 12mer Tg peptide (2549-2560), containing T4 at aa position 2553 – T4(2553) -

has been reported to elicit both proliferative and cytotoxic T-cell responses (Kong et al. 1995b, Hutchings et al. 1992, Wan et al. 1998) and to cause lymphocytic as well as granulomatous EAT (Braley-Mullen and Sharp 1997). Elegant studies by Wan et al. using the thyronine (T0)-containing analog T0(2553) -- thyronine lacks the four iodine atoms of T4 -- found that LNC from mice challenged with the T4(2553) peptide could not be cross-stimulated in vitro with T0(2553), and this regimen failed to generate effector cells that could transfer EAT (Wan et al. 1997). These data suggested, for the first time, that iodine atoms sufficiently modify the peptide-MHC complex to elicit a distinct subset of thyroid-infiltrating T cells that recognize only the iodinated determinant. The presence of the bulky two-phenyl-ring side chain is not sufficient to impart immunogenicity, since other T4-containing peptides were either mildy pathogenic or were devoid of immunogenicity (Wan et al. 1997).

Prompted by the above observations, and by the fact that most of the iodine in Tg is found in iodotyrosyls outside the hormonogenic sites (Dunn and Dunn 2000), we have hypothesized that Tg may harbour several pathogenic T -cell epitopes that contain iodotyrosyls. In this study, we undertook a systematic search of Tg to localize such post-translationally modified neoantigenic determinants that would have escaped detection in earlier mapping studies. Further goals were to examine whether iodine-modified epitopes were present in normal or HI-Tg and whether they comprised immunodominant sites.

3.3 RESULTS

Iodotyrosyls alter the immunogenicity of Tg peptides.

Since I-A^k genes are known to control susceptibility to Tg-induced EAT (Beisel et al. 1982a, Kong et al. 1997, Vladutiu and Steinman 1987), we scanned the complete murine Tg sequence (Kim et al. 1998) for the presence of I-A^k-binding motifs flanked by Tyr residue(s), using the algorithm described by Altuvia *et al.* (Altuvia et al. 1994). Twenty sites meeting these criteria were identified and from these, a total of 13 peptides, ranging in size from 11 to 17 aa residues, were synthesized in their non-iodinated or iodinated, i.e. iodotyrosyl-containing, form (**Table 3.1**).

Table 3.1 Tg peptides encompassing I-A^k-binding motifs flanked by Tyr.

a.a. coordinates [a]	Motif-containing sequence [b]	Peptide synthesized [d]	Peptide denotation
107-117	[Y]APV**QCDLQ**RV [c]		
121-130	**CVDTE**GMEV[Y]	VQCW**CVDTE**GMEV[Y]GT	p117
182-192	DMMIFDLIHN[Y]	NTTDMMIFDLIHN[Y]NR	p179
228-239	ETGL**ELLLD**EI[Y]	LAETGL**ELLLD**EI[Y]DTI	p226
306-316	[Y]QTV**QCQTE**GM	GH[Y]QTV**QCQTE**GMCW	p304
612-620	[Y]AGE**CWCVD**	QC[Y]AGE**CWCVD**SRGK	p610
684-689	[Y]**CVDTE**	SEC[Y]**CVDTE**GQVIP	p681
758-765	HEQVFEW[Y]	PHEQVFEWYERW	p757
841-847	**NIFLDP**[Y] [c]		
1354-1364	DISVGSLPDL[Y] [c]		
1393-1404	DSKTFSADTTL[Y]	LHLDSKTFSADTTL[Y]FL	p1390
1404-1415	[Y]FLNGDSFVTSP [c]		
1935-1942	NDKVNNF[Y]	KVVLNDKVNNF[Y]TRL	p1931
2029-2035	**DTEVHT**[Y]	GSE**DTEVHT**[Y]P	p2026
2135-2143	[Y]PDI**QNCIH** [c]		
2306-2314	NFIVVTAN[Y]	AVGNFIVVTAN[Y]RLG	N/A
2532-2543	DARILAAAVW[YY]	EDSDARILAAAVW[YY]SL	p2529
2542-2552	[YY]SL**EHSTD**D[Y]	VW[YY]SL**EHSTD**D[Y]AS	p2540
2596-2609	[Y]G**HGSLE**LLADVQ[Y]	ES[Y]G**HGSLE**LLADVQ[Y]AFG	N/A
2619-2626	[Y]QG**QFSTE**	SA[Y]QG**QFSTE**EQSL	p2617

[a] Amino acid coordinates of the motif-containing sequence were assigned according to the mouse Tg sequence data by Kim *et al.* (Kim et al. 1998) and do not include the 20 a.a. leader peptide.

[b] The Tg sequence was scanned for the presence of two I-A^k binding motifs, a heptamer motif A (underlined) or a pentamer motif B (bold-face), by using the algorithm of Altuvia et al. (Altuvia et al. 1994). Only peptides with motifs flanked by one or more Tyr residues (in boxes), within 5 aa positions from the N- or C-terminus of the motif, were selected. Motif A: [DEHNQ]-{NQPY}-{PY}-[ILTV]-{PY}-{FHWYP}-[ACFILMTVWP]; Motif B: [CDEHNQ]-{DEPY}-{PY}-[ILTV]-[DEHQN]; [ ] denotes inclusion and {} denotes exclusion of indicated a.a. at this position.

[c] Peptides containing Pro residues within the motif or between Tyr residues and the motif were excluded because of concerns that Pro may drastically affect the secondary structure.

[d] Each peptide was synthesized as a pair of non-iodinated or iodotyrosyl-containing analogs.

[N/A] The iodinated analogs of these peptides could not be synthesized commercially.

To determine their immunogenicity at the T-cell level, CBA/J mice were s.c. challenged with 200 nmol of each peptide analog in adjuvant and nine days later, draining LNC were collected and cultured in the presence of varying concentrations of the respective peptides. Seven out of 13 peptides elicited no response, regardless of their iodination status. Three Tg peptides were immunogenic in their non-iodinated form and iodotyrosyl formation had variable effects, as it increased (p179), decreased (p2540) or did not alter (p2529) their immunogenic profile (See Chapter 4). However, Three peptides - p117, p304, and p1931 - were non immunogenic in their non-iodinated form but their iodotyrosyl-containing analogs induced significant LNC responses (**Fig. 3.1, A,B,C**) accompanied by IL-2 as well as IFN-γ secretion in vitro (**Fig. 3.1, G,H,I**). Furthermore, each iodinated analog activated LNC that did not cross react with the non-iodinated form of the peptide (**Fig. 3.1, D,E,F**), indicating that iodine heavily influenced the immunogenicity as well as the antigenicity of these determinants. This was confirmed at the clonal T-cell level using the hybridomas 4A6, 10C1, and 1H7 which secreted IL-2 upon culture with dendritic cells (DC) presenting I-p117, I-p304, and I-p1931, respectively, but did not recognize equimolar amounts of the non-iodinated analogs (**Fig. 3.2, A, B, C**). All three hybridomas were A^k-restricted $CD4^+$ T cell hybridoma clones (**Fig. 3.3 & 3.4**). The same hybridoma clones were not activated by DC processing intact Tg or HI-Tg, suggesting that the iodinated peptides are not immunodominant in either normal Tg or HI-Tg (**Fig. 3.2, D, E, F**).

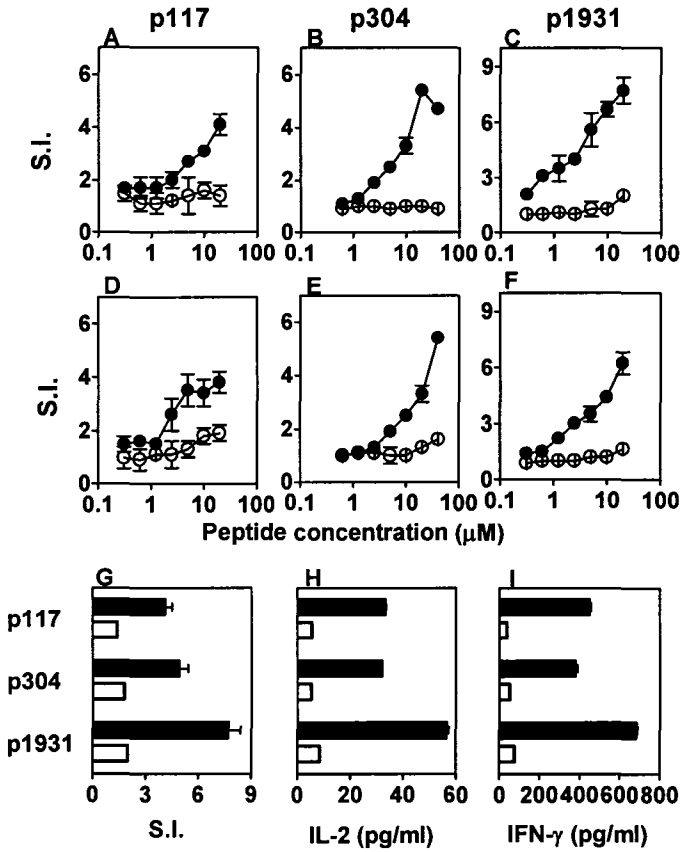

Figure 3.1. Iodotyrosyls impart immunogenicity to Tg peptides.

A,B,C, CBA/J mice (2 mice per group) were primed with the non-iodinated (○) or iodinated (●) form of the indicated peptides and LNC responses were tested, 9 days later, against the respective peptide. **D,E,F,** Proliferative responses of LNC from mice primed with indicated iodinated peptide against the non-iodinated (○) or iodinated (●) form of the priming peptides. **G,H,I,** Determination of proliferative responses (G) and cytokine secretion (**H,I**) in 48-h culture of peptide-primed LNC incubated in the presence of 20 μM of the respective peptide. Results are representative of 2-4 independent groups. IL-4 and IL-10 were undetectable in all experiment groups.

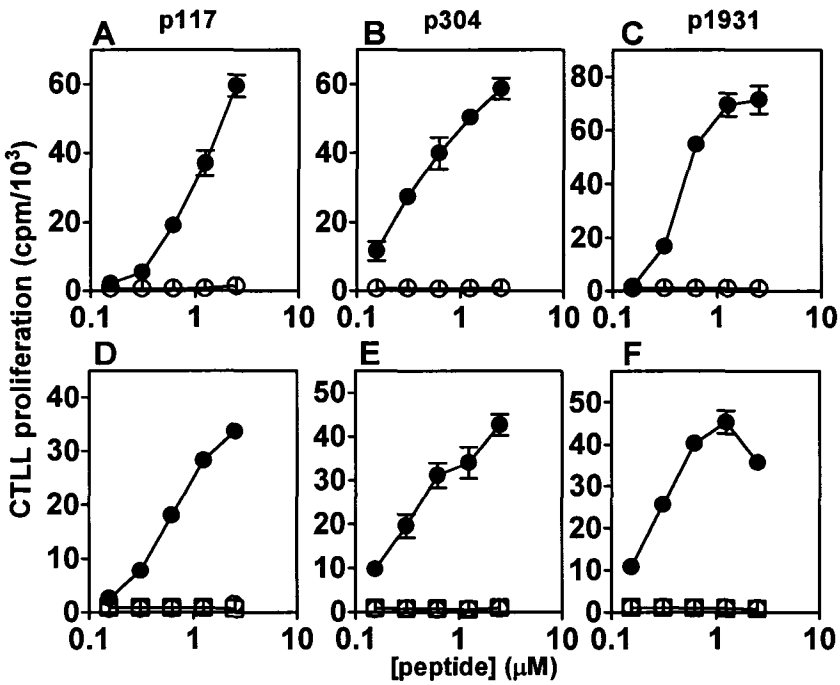

Figure 3.2. T cell hybridoma clones specific for I-p117, I-p304, I-p1931 do not respond to the non-iodinated analogs, intact Tg or HI-Tg.

A,B,C, IL-2 secretion by the T cell hybridoma clones 4A6, 10C1, and 1H7 cultured in the presence of DC and iodinated (●) or non-iodinated (○) forms of p117, p304, and p1931, respectively. **D,E,F,** Activation of the T cell hybridoma clones 4A6, 10C1, and 1H7 by its ligand I-p117, I-p304, I-p1931, as well as intact Tg (□) and HI-Tg (○). Data are representative of two to four independent experiments. Background values ranged from 2000-5000 cpm.

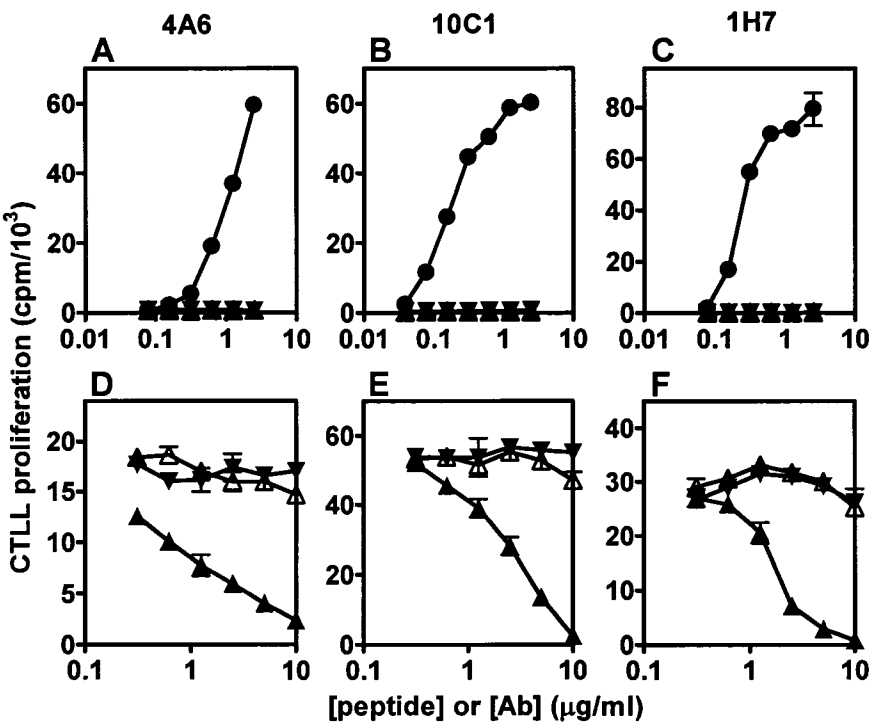

Figure 3.3. MHC restriction of T cell hybridoma clones 4A6, 10C1 and 1H7.

A,B,C, IL-2 secretion upon activation of the 4A6, 10C1 and 1H7 T cell hybridoma clones, by its ligand I-p117, I-p304 and I-p1931 (•), respectively, as well as by anti-A^k (▲), anti-E^k (▼) and control (Δ) antibodies, using LK35.2 as APC. **D,E,F**, Activation of 4A6, 10C1 and 1H7 clones in the presence of 0.5 μM of its specific ligand and increasing concentrations of the anti-A^k (▲), anti-E^k (▼) and control (Δ) antibodies.

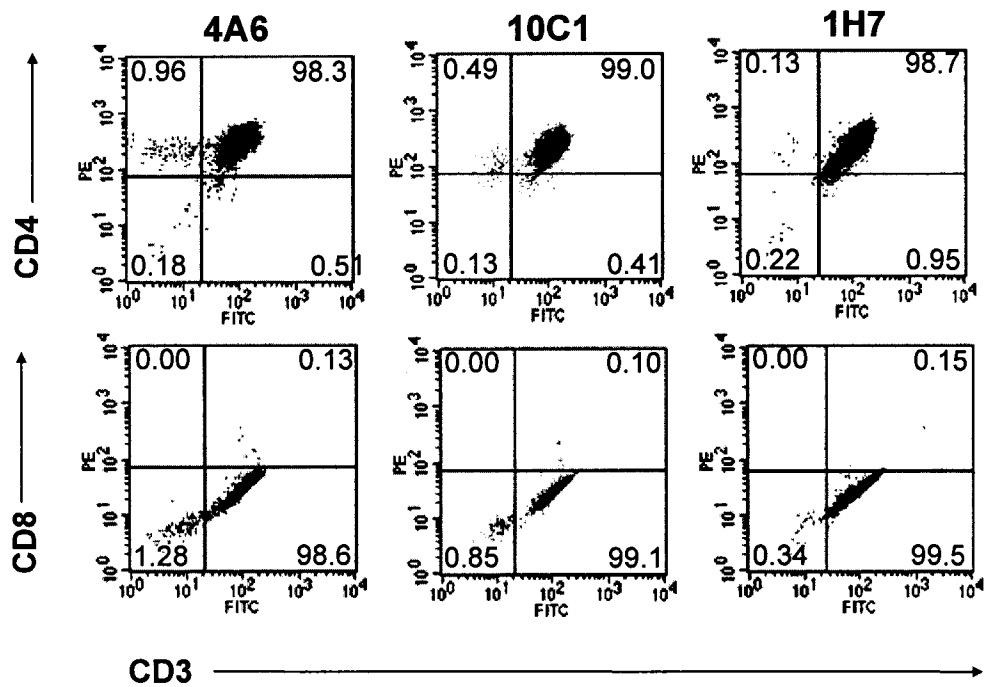

Figure 3.4 Phenotypic analysis of surface markers on the T cell hybridoma clones 4A6, 10C1 and 1H7.

Expression of T cell surface markers on the 4A6.3, 10C1.8 and 1H7.16 T cell hybridoma clones was determined by FACS. T cell hybridoma clones were harvested and double-stained with FITC-anti-CD3 mAb, and PE-conjugated mAbs against CD4 or CD8.

Iodotyrosyl formation promotes peptide-binding to MHC or TcR recognition of the peptide-MHC complex

To investigate whether iodine enhanced the immunogenicity of these peptides by promoting peptide binding to MHC, we performed a competition assay using the 4A12 T-cell hybridoma clone which was previously (Rao et al. 1994) shown to be A^k-restricted, and reactive against the p2498 (aa 2498-2506) epitope of Tg (**Fig. 3.5 A**). The 4A12 cells were activated with 1 μM p2498 in the presence of increasing concentrations of inhibitor peptides using the LK35.2 (A^k-expressing) antigen presenting cell (APC) line. It was observed that p117 and p304 could not inhibit 4A12 activation at the 10-100 μM range (**Fig.3.5, B, C**) whereas their iodinated analogs, at equimolar concentrations, significantly diminished 4A12 activation. These results suggested that p117 and p304 are not A^k-binders and that iodotyrosyl formation within these sequences promoted their binding to the A^k molecule, leading to enhancement of their immunogenicity in CBA/J mice. In contrast, both the iodinated and non-iodinated analogs of p1931 significantly inhibited the activation of 4A12 (**Fig. 3.5 D**) suggesting that, in this case, the iodotyrosyl side chain must face away from the MHC cleft and make direct contact with the TcR. The enhanced immunogenicity of I-p1931 would result from recruitment of T cells able to recognize only the iodine-modified peptide-MHC complex.

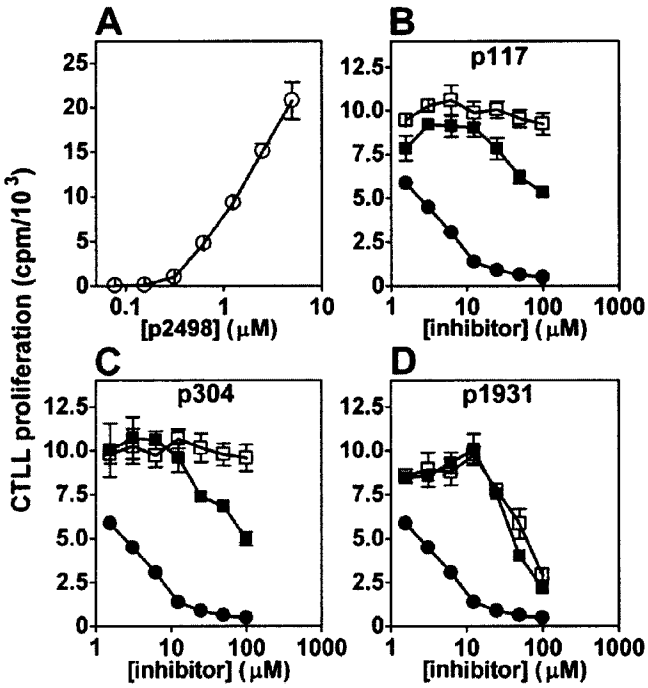

Figure 3.5. Iodotyrosyl formation promotes peptide binding to MHC or TcR recognition of the peptide-MHC complex.

A, IL-2 secretion upon activation of the 4A12 T-cell hybridoma (A^k-restricted), by its ligand p2498 using LK352 cells as APC. **B,C,D,** Activation of 4A12 in the presence of 1 M p2498 and increasing concentrations of the inhibitor peptides shown in their iodinated (■) or non-iodinated (□) form. The mouse lysozyme peptide 46-62 (●), known to bind to A^k, was used as a positive control.

EAT and Ab responses induced by iodotyrosl-containing Tg peptides

The pathogenicity of I-p117, I-p304, and I-p1931 was subsequently tested by direct challenge of CBA/J mice (6 mice per group) with the corresponding peptide in adjuvant or by adoptive transfer of peptide-primed LNC into naïve CBA/J hosts. I-p117 and I-p1931 elicited thyroiditis by either experimental protocol, although the severity of EAT was higher by the adoptive transfer method (mean infiltration index of 0.67 vs 2.33 for I-p117, and 0.83 vs 1.67 for I-p1931) (**Table 3.2 and Fig. 3.6**). I-p304 was thyroiditogenic only by the adoptive transfer method (mean infiltration index of 0.83). At the B-cell level, only I-p117 was strongly immunogenic eliciting serum IgG responses to itself and native Tg (**Table 3.2**). There was no cross reactivity with the non-iodinated analog, suggesting that I-p117 is localized at the surface of the intact Tg molecule. Interestingly, previous studies have shown that the Tyr residue at position 130 is an early iodination site in Tg (Lamas et al. 1989).

Table 3.2 EAT and Ab responses induced by iodotyrosyl-containing Tg peptides

Priming peptide	Induction of EAT								Serum IgG response c @ 1:30 dilution (OD 405 nm) against		
	Mode of challenge	Infiltration Index (I.I.) 0	1	2	3	4	5	Mean	# of mice with EAT	Priming peptide	Non-iodinated analog	Tg
I-p117	Direct [a]	3	2	1	0	0	0	0.67	3/6	1.321 + 0.061	0.159 + 0.014	0.547 + 0.020
	AT [b]	2	0	1	1	1	1	2.33	4/6			
I-p304	Direct [a]	6	0	0	0	0	0	0.00	0/6	0.318 + 0.006	0.147 + 0.001	0.167 + 0.003
	AT [b]	3	1	2	0	0	0	0.83	3/6			
I-p1931	Direct [a]	3	2	0	1	0	0	0.83	3/6	0.169 + 0.001	0.154 + 0.001	0.139 + 0.004
	AT [b]	2	0	2	2	0	0	1.67	4/6			

[a] CBA/J mice were s.c. challenged with 100 nmol of the indicated peptide in CFA and boosted, 3 weeks later, with 50 nmol of the same peptide in IFA. EAT was assessed 35 days after the initial challenge.

[b] EAT elicited by the adoptive transfer (AT) of 2×10^7 peptide-primed LNC into naïve syngeneic hosts. Thyroid pathology was assessed 14 days post-transfer.

[c] Immune sera were obtained on day 35 from mice challenged as in (A). ELISA results are expressed as mean OD values of duplicate wells $\pm$ SD.

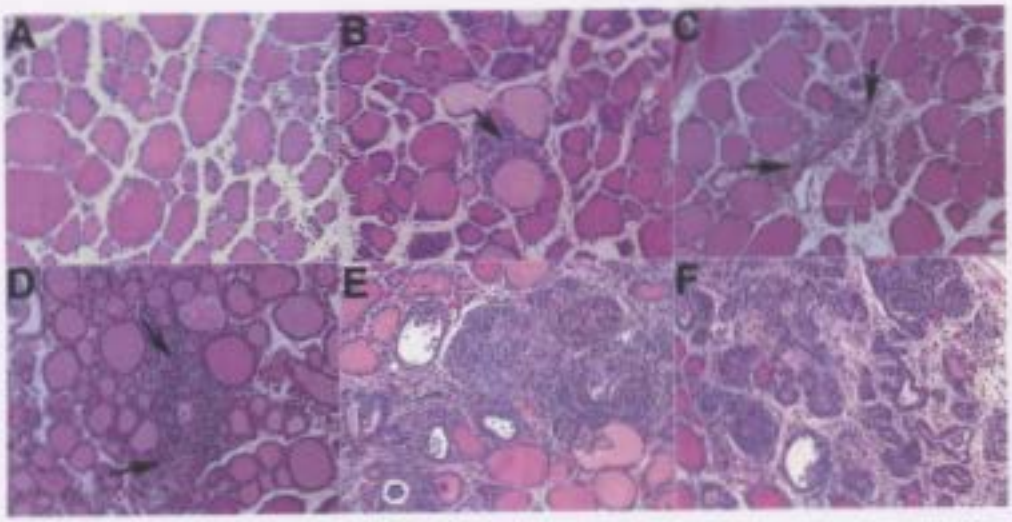

Figure 3.6 Histological appearance of EAT elicited by iodotyrosyl-containing Tg peptides.

A, Normal gland, I.I. = 0. **B,** Interstitial accumulation of inflammatory cells (arrows); I.I. = 1. **C,** One or two foci of inflammatory cells (arrows); I.I. = 2. **D,** Diffuse infiltration, 10–40% of total area (arrows); I.I.= 3. **E,** Extensive infiltration 40-80% of total area; I.I. = 4. **F,** Extensive infiltration, more than 80% of total area. Magnification: x 200.

3.4 DISCUSSION

The iodine content of Tg varies widely (0.1 – 1.0% of weight) with iodine availability, but on average, 19 out of the 26 atoms of iodine within each 19S dimer are stored in the form of mono- and di-iodotyrosines (Dunn and Dunn 2000). The present findings demonstrate that iodotyrosyls at residues 130, 306, and 1942 contribute to the formation of neoantigenic T-cell epitopes which elicit EAT in CBA/J mice. These data and the lack of antigenicity of the non-iodinated analogs p117, p304 and p1931 further suggest that Tg harbors iodotyrosyls at these residues under normal, steady-state conditions. Despite their non-dominant nature, these peptides must be generated intrathyroidally and be recognized by the adoptively transferred effector cells mediating EAT. Thus, the current and earlier epitope mapping studies (Carayanniotis 2003) delineate at least three distinct categories of T-cell determinants with pathogenic potential in Tg: a) T4-containing peptides in which iodine atoms are an integral part of the TcR ligand (Dai et al. 2005, Dawe et al. 1996, Hutchings et al. 1992, Kong et al. 1995b); b) iodotyrosyl – containing peptides, as described herein; and c) non-iodinated epitopes (Carayanniotis 2003). It is yet unknown whether di-iodotyrosyls contribute in the formation of determinants that elicit EAT.

Formation of pathogenic Tg epitopes by iodine presents another example of post-translational modifications associated with autoimmune responses at both the T-or B-cell levels (Anderton 2004, Doyle and Mamula 2001). The bulky iodine atoms (atomic radius of ~133 pm) in the iodotyrosyl side chain can be critical in forming a TcR-contact

residue, as suggested by the results with the I-p1931 peptide herein, and by earlier studies showing that iodine atoms on the longer thyroxyl side chain in T4(2553) influence its recognition by clonal or polyclonal T cells (Dai et al. 2005, Kong et al. 1995b). In addition, the findings with the I-p117 and I-p304, highlight, for the first time, that iodotyrosyl formation facilitates peptide binding to MHC. Specific recognition of iodine-modified Tg epitopes by B cells has been suggested by earlier studies (Saboori et al. 1998) and is demonstrated by the observation that I-p117-specific IgG does not bind to p117. The Y130 residue within I-p117 was reported as an early iodination site in Tg (Lamas et al. 1989) but this was not confirmed by another study (Xiao et al. 1996). Recognition of iodine-modified Tg peptides by antibodies has been also noted even following formation of peptide-MHC complexes. For example, a mAb recognizing the 5' iodine atom of the outer phenolic ring of T4 has been shown to inhibit T-cell recognition of the T4(2553)-MHC complex (Dai et al. 2005).

Enhanced iodotyrosyl formation, catalyzed by thyroid peroxidase (Dunn and Dunn 2000), may account, in part, for the increased immunogenicity of highly iodinated Tg (Champion et al. 1987, Dai et al. 2002, Saboori et al. 1998, Sundick et al. 1987). At the level of individual determinants, however, iodotyrosyls not only contribute to the formation of neoantigenic epitopes, but may have neutral or immunomodulatory effects as shown by the p179, p2529, and p2540 results (Chapter 4). Iodine atoms per se do not necessarily impart immunogenicity, as exemplified by I-p681 and I-p757 which encompass early iodination sites (Xiao et al. 1996), but are not immunogenic. In addition, the non-

iodinated Tg peptide (306-320) was previously identified as pathogenic (Verginis et al. 2002) whereas the overlapping non-iodinated (304-318) fragment was not immunogenic in this study, possibly reflecting effects of flanking residues on recognition by TcR. These effects will be better understood once the relative position of iodotyrosyl vis a vis the minimal T cell epitopes within those sequences is delineated.

The iodine content of Tg is known to affect its structure (Edelhoch et al. 1969) as well as its proteolytic degradation (Fouchier et al. 1983, Lamas et al. 1989, Lamas and Ingbar 1978) and altered processing of highly iodinated Tg in APC has been shown to facilitate generation of cryptic non-iodinated pathogenic determinants, such as the p2494 peptide (Dai et al. 2002). The I-p117, I-p304, and I-p1931 peptides are not generated by the processing of highly iodinated Tg in DC (**Fig3.2, D, E, F**), but they could be generated following processing of Tg-Ab immune complexes, as has been shown for other non-iodinated determinants (Dai et al. 1999). Lastly, enhanced iodination of Tg may exert immunomodulatory influences as it has been reported that removal of iodine from tryptic human Tg fragments converts them from immunogenic to tolerogenic (Gardine et al. 2003). Our results demonstrate the existence of a new group of pathogenic Tg determinants which cannot be detected by conventional mapping methods. While their potential role in the development of clinical disease remains to be elucidated, they provide a new insight as to how an environmental trigger (iodine supply) may influence the development of thyroid disease.

CHAPTER 4

MODIFYING EFFECTS OF IODINE ON THE IMMUNOGENICITY OF THYROGLOBULIN PEPTIDES

(This is a modified version of manuscript submitted for publication)

Haiyan S. Li, Hong Jiang and George Carayanniotis

Divisions of Endocrinology and Basic Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

4.1 ABSTRACT

We have previously shown that iodotyrosyl formation within thyroglobulin (Tg) generates neoantigenic determinants that are immunopathogenic. In the current study, we have examined iodination effects on three tyrosyl-containing Tg peptides that are immunogenic in their non iodinated form. We found that iodotyrosyl formation can enhance (p179, aa. 179-194), suppress (p2540, aa. 2540-2554), or not alter (p2529, aa. 2529-2545) the immunogenic profiles of these peptides at the T- cell level. On the other hand, iodination did not alter the MHC-restriction profile of p2529 and p2540 (A^k-binders) or p179 (A^k- and E^k-binder) and did not significantly influence the pathogenicity of these determinants. At the B-cell level, addition of an iodine atom on Y192 in p179 generated a neoantigenic determinant, but analogous effects were not discernible in p2529 or p2540. Our results demonstrate that iodotyrosyl formation can exert variable effects on the immunogenic behavior of Tg epitopes which may not always result in enhanced pathology. These findings also suggest that variations in the iodine content of Tg may significantly alter the hierarchy of antigenic determinants, to which the immune system may or may not be tolerant.

4.2 INTRODUCTION

Iodide ingestion has been well known to promote development of experimental autoimmune thyroiditis (EAT) in autoimmune-prone animals, such as obese-strain chickens (Bagchi et al. 1985), BB/W rats (Allen et al. 1986) and NOD.H-2^{h4} mice (Rasooly et al. 1996). Since the iodine content of thyroglobulin (Tg) varies widely (0.1-1% of weight) according to the environmental supply of this element (Dunn and Dunn 2000), it has been hypothesized that the iodination level of Tg is pivotal in precipitating autoimmune processes leading to disease. Indeed, it has been well documented that the post-translational modification of Tg by iodide affects not only hormone synthesis (Dunn et al. 1983, Dunn and Dunn 2000), but also the immunopathogenicity of this large autoantigen. Thus, the iodine content in normal Tg has been shown to be essential for induction of Tg-specific T- and/or B-cell responses as well as thyroid lesions in humans and experimental animals (Champion et al. 1987, Ebner et al. 1992, Rasooly et al. 1998). In addition, enhanced iodination of Tg has been reported to augment its immunoreactivity and disease-causing potential (Champion et al. 1987, Dai et al. 2002, Saboori et al. 1998, Sundick et al. 1987).

Progress in Tg epitope mapping has begun to shed light on the molecular understanding of these observations. Tg harbors at least three distinct subsets of pathogenic T-cell determinants: a) thyroxine (T4)-containing epitopes in which iodine atoms within the T4 structure are an integral part of the T cell receptor (TcR) ligand (Dai et al. 2005, Dawe et al. 1996, Hutchings et al. 1992, Kong et al. 1995a); b) determinants that become

immunopathogenic only after incorporation of iodine into iodotyrosyl residues (Li and Carayanniotis 2006); and c) non iodinated epitopes (Carayanniotis 2003). Enhanced iodine uptake by Tg can be viewed to promote formation of neoantigenic determinants in (a) and (b), but also it may facilitate generation of pathogenic non iodinated epitopes via its effects on Tg processing in antigen presenting cells (APC) (Dai et al. 2002). In this study, we have examined what effects iodotyrosyl formation may have within Tg peptides with established pathogenicity. To this end, we have focused on three Tyr-containing peptides, p179 (aa 179-194), p2529 (aa 2529-2545) and p2540 (aa 2540-2554) identified in an earlier study (Li and Carayanniotis 2006) which are immunopathogenic in their non iodinated form.

4.3 RESULTS

Iodotyrosyls modify the antigenicity and immunogenicity of Tg peptides

As described in our previous report (Li and Carayanniotis 2006), thirteen Tg peptides, encompassing A^k-binding motifs (Altuvia et al. 1994) flanked by Tyr residue(s), were synthesized as analog pairs, representing the non iodinated and iodinated, i.e. iodotyrosyl-containing, form. Among them, three peptides - p179, p2529 and p2540 (aa sequences shown in **Table 4.1**) containing one, two or three tyrosyls, respectively - were immunogenic in their non iodinated form, as they induced marked specific LNC proliferation following their administration in CBA/J mice (**Fig. 4.1A-C**). However, iodotyrosyl formation had variable effects on their antigenicity since the same LNC populations cross-reacted either weakly against the iodinated analogs (responses to I-p179 and I-p2540, **Fig. 4.1A and C**) or equally well (response to I-p2529, **Fig. 4.1B**). When the iodinated analogs were used as priming antigens, it was again found that iodine variably modified their immunogenic profile. Thus, I-p179 elicited significantly stronger LNC responses than p179 (S.I. = 21.2 $\pm$ 1.3 vs 15.0 $\pm$ 0.7 at 20 μM) (**Fig. 4.1A and D**), I-p2540 was less immunogenic than p2540 (S.I. = 4.54 $\pm$ 0.5 vs 9.7 $\pm$ 0.9 at 20 μM) (**Fig. 4.1 C and F**) and I-p2529 elicited equivalent responses to those obtained by p2529 (S.I. = 13.7 $\pm$ 0.9 vs 13.9 $\pm$ 1.0 at 20 μM) (**Fig. 4.1B and E**). LNC primed with the iodinated analogs were again variably cross-reactive with the respective non-iodinated peptides (**Fig. 4.1D-F**). These data highlighted the fact that addition of iodine atoms to Tg peptides may have variable effects on both their antigenic and immunogenic behavior.

Table 4.1 The a.a. sequence of Tg peptides used in this study.

a.a. coordinates [a]	Motif-containing sequence [b]	Peptide synthesized [c]	Peptide denotation
181-192	**TDMMIFDL**IHN[Y]	NT**TDMMIFDL**IHN[Y]NR	p179
2532-2543	DARIL**AA**AVW[YY]	EDSDARIL**AA**AVW[YY]SL	p2529
2542-2552	[YY]SLEHSTDD[Y]	VW[YY]SLEHSTDD[Y]AS	p2540

[a] Amino acid coordinates of the motif-containing sequence were assigned according to the mouse Tg sequence with Swiss-Prot accession number **O08710** (http://ca.expasy.org/uniprot/O08710) and do not include the 20 a.a. leader peptide.

[b] Based on the algorithm of Altuvia et al. (Altuvia et al. 1994), Tg peptides were selected based on the presence of A^k -binding heptamer or pentamer motifs (underlined). Two out of the three peptides also encompass overlapping heptamer or pentamer E^k-binding motifs (**bold face**).

[c] Each peptide was synthesized as a pair of non iodinated and iodotyrosyl-containing analogs.

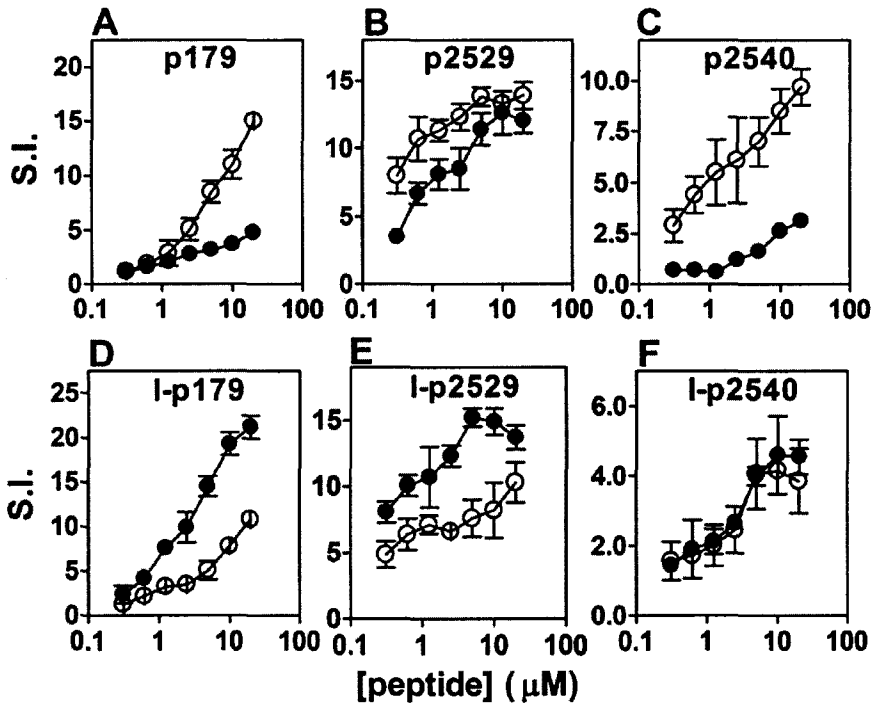

Figure 4.1. Iodotyrosyls variably modify the immunogenicity of Tg peptides.

CBA/J mice (2 mice per group) were primed with the non iodinated (**A-C**) or iodinated (**D-F**) peptide as indicated and LNC responses were tested, 9 days later, against the respective non iodinated (○) or iodinated (●) forms of these peptides. Data are representative of two to four independent experiments. Background values ranged from 2000-5000 cpm.

Iodotyrosyl formation in Tg peptides does not alter their MHC-restricted recognition by T cells

The above Tg peptides were initially identified as putative A^k-binders, but further analysis indicated that p179 and p2529 also encompassed an overlapping E^k-binding motif (**Table 4.1**). To investigate the MHC context in which these peptides were presented and whether iodination could change their MHC-binding pattern, peptide-specific LNC recall assays were performed in the presence of titrated concentrations of A^k-, E^k-, or NP (control) - specific mAbs. The proliferation of p179-reactive LNC was blocked by E^k-specific mAbs (% inhibition = 84.17 $\pm$ 4.14), and to a lesser extent by mAbs against A^k (% inhibition = 47.18 $\pm$ 12.44) (**Fig. 4.2**), suggesting the presence of non overlapping p179-reactive T cell subsets restricted by either MHC molecule. In contrast, for p2529 and p2540, LNC proliferation was significantly blocked by A^k-specific mAbs (% inhibition = 59.75 $\pm$ 3.45, 80.87 $\pm$ 3.7, respectively), whereas anti-E^k mAbs mediated only background inhibition similar to that observed by anti-NP mAbs. Interestingly, the inhibition pattern of proliferation was very similar for the two analogs of each pair strongly suggesting that, at least for these three peptide pairs, iodination was not sufficient to alter the MHC-restriction profile.

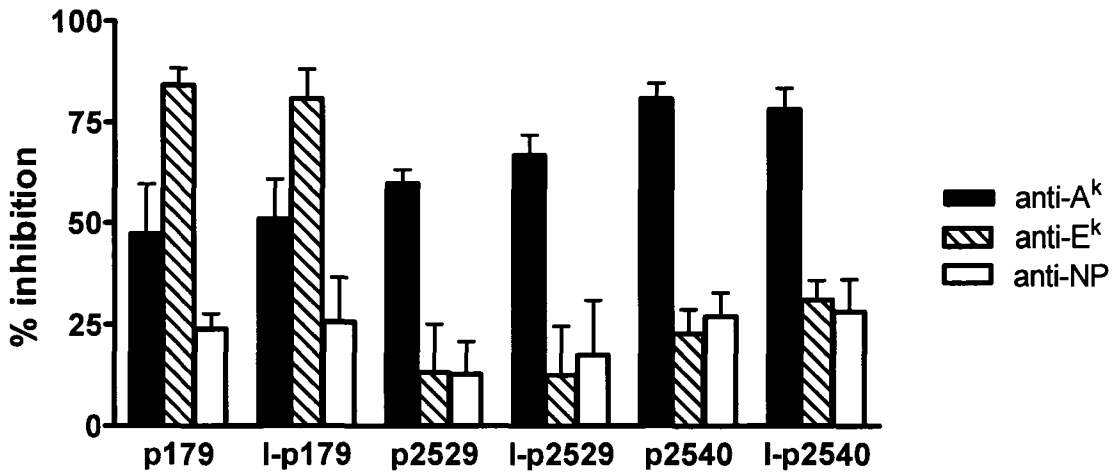

Figure 4.2. Iodination does not alter the MHC-restricted recognition of immunogenic tg peptides by T cells.

Following priming of CBA/J mice with the indicated peptide, draining LNC were cultured in the presence of either non iodinated or iodinated p179, p2529 or p2540 at a final concentration of 10 μM, 2.5μM or 10 μM, respectively. Serial dilutions of mAbs specific for A^k, E^k or influenza NP (control) were added to the culture at the concentrations from 10 to 0.157 μg/ml. The % inhibition data express mean ± SD values of triplicate wells at 5 μg/ml of mAb and are representative of three experiments.

EAT and Ab responses induced by Tg peptides and their iodinated analogs

The thyroiditogenicity of the three peptide analog pairs was subsequently tested in CBA/J mice (6 mice per group), following challenge with each respective peptide emulsified in CFA. Each of the six peptides induced very mild EAT in less than half of experimental animals, with mean infiltration index ranging from 0.17 to 0.67, respectively (**Table 4.2 & Fig. 3.6**). Interestingly, the incidence and severity of EAT induced by the iodinated analogs were not significantly different from those elicited by the non iodinated peptides, suggesting that alterations in immunogenicity do not always correlate with the histopathological picture. At the B-cell level, p2529, p2540, and their iodinated analogs induced significant specific IgG responses which were strongly cross-reactive within each pair (**Table 4.2**). The presence of the iodine atom seemed either not to influence the antigenicity or immunogenicity of the peptide (p2529) or to diminish it (p2540). p179 was not immunogenic, but interestingly, iodination of Y192 created a new B-cell epitope, which elicited strong IgG responses against both I-p179 and p179 ($OD_{405\ nm}$ = 1.865 $\pm$ 0.138, 1.790 $\pm$ 0.077, respectively). There was no detectable IgG reactivity against intact Tg in all groups, suggesting that these peptides are not likely localized on the Tg surface to be accessible by Abs.

Table 4.2. Immunopathogenic properties of Tg peptides in CBA/J mice.

Priming peptide	Induction of EAT [a]					Serum IgG response [b] @ 1:240 dilution (OD 405 nm) against		
	Infiltration Index (I.I.)				# of mice with EAT	Non iodinated analog	Iodinated analog	Tg
	0	1	2	Mean				
p179	3	2	1	0.67	3/6	0.207 ± 0.009	0.166 ± 0.008	0.165 ± 0.006
I-p179	4	1	1	0.50	2/6	1.790 ± 0.077	1.865 ± 0.138	0.170 ± 0.003
p2529	5	1	0	0.17	1/6	3.640 ± 0.071	3.503 ± 0.087	0.181 ± 0.002
I-p2529	5	0	1	0.33	1/6	3.546 ± 0.077	3.632 ± 0.093	0.194 ± 0.007
p2540	3	1	2	0.50	3/6	0.986 ± 0.052	0.736 ± 0.014	0.202 ± 0.004
I-p2540	5	0	1	0.33	1/6	0.544 ± 0.045	0.620 ± 0.069	0.164 ± 0.011

[a] Mice were s.c. challenged with 100 nmol of the indicated peptide in CFA and boosted, 3 weeks later, with 50 nmol of the same peptide in IFA. EAT was assessed 35 days after the initial challenge.

[b] Immune sera were obtained on day 35 from mice challenged as in (a). ELISA results are expressed as mean OD values of duplicate wells ± SD.

Dominance/Crypticity of the immunogenic Tg peptides

To characterize the dominance and crypticity of the Tg immunogenic peptides described in Chapter 3 and herein, CBA/J mice were immunized with Tg or Tg peptides in CFA and, 9 days later, draining LNC were collected. Generation of the Tg peptide following processing of intact Tg in vivo (**Fig. 4.3A**) or in vitro (**Fig. 4.3B**) was tested by a proliferative LNC assay. Tg-primed LNC responded in vitro significantly to the intact protein at 1.25 μM, as well as to free p179 at higher doses (20 μM), but not to other peptides (**Fig. 4.3 A**). Meanwhile, p179-primed LNC responded to the priming peptide and intact Tg at equimolar concentrations (1.25 μM) (**Fig. 4.3 B**). In contrast, LNC primed with other peptides proliferated in vitro against the priming peptide, but not intact Tg. Therefore, we conclude that among all the newly identified immunogenic Tg peptides, p179 encompasses a subdominant epitope.

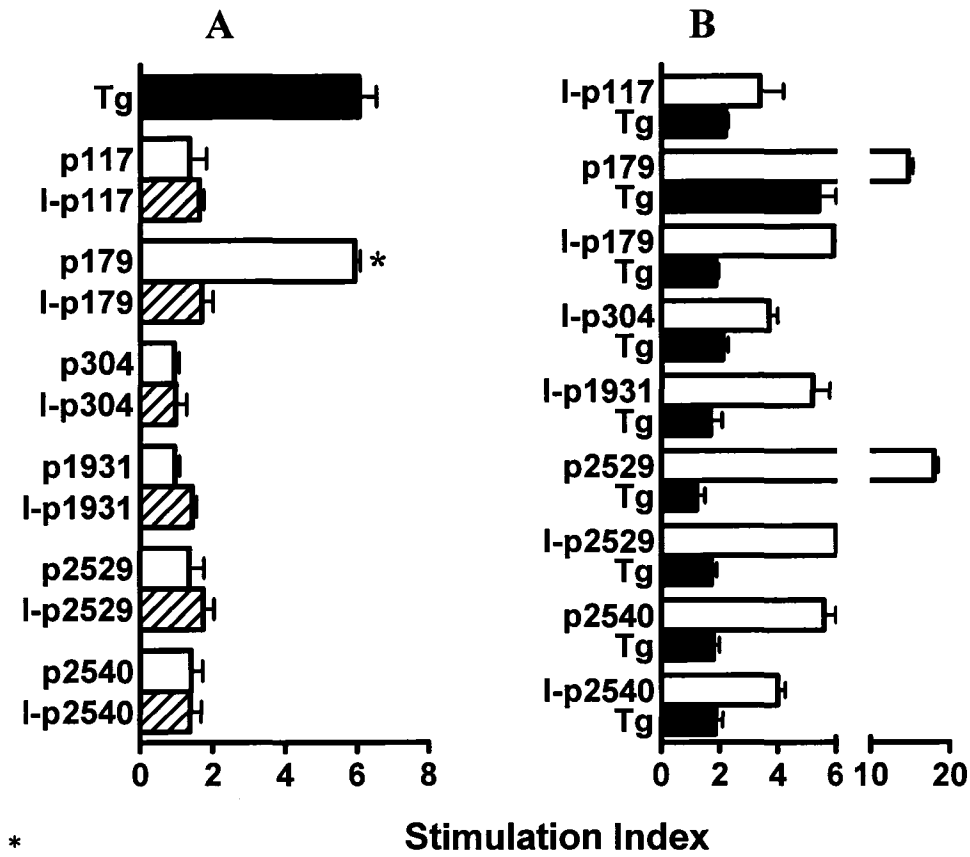

Figure 4.3. p179 contains a subdominant Tg epitope.

A, CBA/J mice (3-6 mice per group) were immunized with Tg and, 9 days later, LNC proliferation was examined in the presence of 1.25 μM of Tg (filled bars), and 20 μM of non-iodinated (open bars) or iodinated (striped bars) Tg peptides, respectively. **B**, Primed LNC from CBA/J mice immunized with Tg peptides were tested against their priming peptide (open bars) and intact Tg (filled bars), both at a concentration of 1.25 μM. Data show the mean ± SD of S.I. values of triplicate wells and are representative of 2-3 experiments. Background cpm varied from 3000 to 6000. * Data are in courtesy of Ms. Hong Jiang, a M.Sc. student in the laboratory.

4.4 DISCUSSION

It has been previously shown that iodotyrosyl formation imparts immunopathogenicity to intact but poorly iodinated Tg (Champion et al. 1987, Ebner et al. 1992, Rasooly et al. 1998) or innocuous Tg peptides (Li and Carayanniotis 2006), at both the T- and B-cell levels. The major aim of the present study was to examine modifying effects of iodine on three Tyr-containing Tg determinants - p179, p2529 and p2540 - that are immunogenic in their non iodinated form. At the T-cell level, addition of iodine to Y192 enhanced the immunogenic properties of p179, but iodotyrosyl formation at Y2542 and Y2543 did not alter the immunogenicity of p2529. Furthermore, simultaneous iodination of Y2542, Y2543, as well as Y2552 – a major hormonogenic site (Malthiery and Lissitzky 1987), diminished the immunogenicity of p2540 at both the T- and B-cell level. These results demonstrate that iodination of individual Tg determinants can either enhance or suppress their immunogenicity. Iodination of the Tg molecule has a net enhancing effect on its immunogenicity (Ebner et al. 1992, Rasooly et al. 1998, Champion et al. 1987, Dai et al. 2002, Saboori et al. 1998, Sundick et al. 1987) because the relative number of neoantigenic determinants may increase (Li and Carayanniotis 2006) or some cryptic but non iodinated peptides may be generated (Dai et al. 2002).

A total of 67 Tyr residues in human Tg are mainly distributed among the three families of cysteine-rich repetitive structures and a region near the C-terminal end of the molecule (Malthiery and Lissitzky 1987), but only a few of them are subject to chemical or enzyme-mediated iodination (Lamas et al. 1989, Xiao et al. 1996). The three peptides

presented herein harbor four Tyr residues, one (Y192) localized within the I.3 domain and three (Y2542, Y2543, Y2552) clustered in the C-terminal region. Y2552 is a major T4 acceptor site, mediating the early formation of mono- or di-iodotyrosine when the availability of iodine is low (Lamas et al. 1989, Xiao et al. 1996). The two phenyl-ring side chain of T4 at this site is an integral part of the (2549-60) epitope recognized by A^k-restricted, Tg-induced hybridomas, because they do not react to the Y2552-carrying analog (Champion et al. 1991). This observation and our current finding that p2540 is mildy thyroiditogenic in CBA mice, strongly argue for the presence of an additional overlapping T–cell epitope within the (a.a. 2540-2554) sequence. Iodination of the Y2542 and Y2543 residues diminish the antigenicity of this epitope. In contrast, the four iodine atoms within T4 promote the immunopathogenicity of (2549-60) although they are not essential for it (Kong et al. 1995b). Thus, iodine atoms may have variable immunomodulatory effects on Tg fragments depending on their positioning within a T-cell epitope.

The peptides described herein encompass either A^k-binding motifs (p2540), or overlapping A^k and E^k- binding motifs (p179 and p2529), and are presented in the context of A^k (p2529 and p2450) or E^k (p179). These results reaffirm the predictive value of the algorithm by Altuvia *et al.* (Altuvia et al. 1994). We have previously shown that iodotyrosyls flanking such motifs can facilitate MHC binding of certain Tg peptides (Li and Carayanniotis 2006). In this study, iodination of p179, p2529 and p2549 does not seem to alter the MHC context in which these immunogenic determinants are presented and recognized by T cells. In this case, it is likely that the bulky iodine atoms contribute

to the formation of critical TcR-contacting iodotyrosyl residues, as suggested previously for the Y1942 within I-p1931 (Li and Carayanniotis 2006), with unpredictable – i.e. enhancing, neutral or suppressive - effects on immunogenicity. Iodination also seems to influence the subdominant nature of the p179 peptide (**Fig. 4.3**), overlapping with the human sequence (aa 181-195) that has been reported to be immunogenic and activate human Tg-primed splenic cells in HLA-DR3 transgenic mice (Flynn et al. 2004b).

The iodine content of normal Tg varies according to its environmental supply (Dunn and Dunn 2000, Sundick et al. 1987) and alters the Tg immunoreactivity by modifying its 3-D structure (Berg and Ekholm 1975, Edelhoch et al. 1969) and stability to enzymatic degradation (Lamas et al. 1989, Xiao et al. 1996). In terms of peptide antigenicity it is, therefore, plausible to see diminishing effects of iodination at the T-cell epitope level and simultaneous enhancing effects at the B- cell epitope level as is the case for p179 (**Fig. 1A and Table 2**). The unpredictable effects iodination may have at a single epitope level have been also highlighted by the studies of Saboori *et al.* (Saboori et al. 1998) in which highly iodinated Tg was not recognized by a mAb (133B1) reactive to non iodinated or normal Tg. Our results imply that the variable iodine content of Tg may alter the hierarchy of available antigenic determinants. How peripheral tolerance mechanisms control homeostasis to an autoantigen like Tg, that is continuously and differentially modified by iodine, remains a fascinating but mostly unexplored area of research.

CHAPTER 5

INDUCTION OF GOITROUS HYPOTHYROIDISM BY DIETARY IODIDE IN SJL MICE

(This is a modified version of manuscript prepared for publication)

Haiyan S. Li and George Carayanniotis

Division of Endocrinology and Basic Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

5.1 ABSTRACT

Prolonged intake of large amounts of iodide has been reported to increase the incidence of goiter and/or hypothyroidism in humans, as well as in animals prone to EAT. In the current study, we have investigated the role of dietary iodide on the development of hypothyroidism, as well as thyroiditis, in strains of mice which do not develop spontaneous thyroiditis. Intake of NaI via drinking water for 10 weeks induced hypothyroidism in SJL/J but not CBA/J mice, as indicated by elevated TSH and depressed total T4 values in serum, as well as formation of colloidal goiter with an inactive flattened thyroid epithelium. Hypothyroidism did not appear to have an autoimmune basis since only focal mononuclear cell infiltrates were found intrathyroidally, and anti-thyroglobulin Abs or increased organification of iodide were not detected. These phenomena were not observed in similarly treated CBA/J mice suggesting polymorphisms in genes controlling events downstream of iodide uptake by thyrocytes. Interestingly, Tg-specific, but not ovalbumin (OVA)-specific IgG responses were suppressed in both SJL and CBA/J mice challenged with Tg following iodide administration, indicating selective immunosuppressive effects of iodide on the response to thyroid antigen(s). Our results point to the generation of a mouse model for the study of iodide-induced hypothyroidism which does not seem to have an autoimmune basis.

5.2 INTRODUCTION

Endemic iodide goiter was previously reported in Japanese populations consuming iodine-rich seaweed (Suzuki et al. 1965) and in Chinese communities drinking water with high iodine contents (Li et al. 1987, Zhao et al. 1998), yet both populations had normal thyroid function. Large quantities of iodide ingestion have also been linked to development of hypothyroidism, assessed by elevated serum TSH concentrations, in schoolchildren (Gao et al. 2004), elder subjects (Laurberg et al. 1998, Szabolcs et al. 1997) and healthy adults (Khan et al. 1998, Konno et al. 1993, Teng et al. 2006). In some studies, when the iodine intake was restricted, the increased TSH levels returned to normal in the absence of anti-thyroid antibodies (Konno et al. 1993). These observations, and several instances of iodide-induced hypothyroidism in patients with underlying thyroid disease or with chronic non thyroidal illnesses (Markou et al. 2001), have strongly suggested that excessive iodine intake may promote the development of hypothyroidism. However, in many studies it remained inconclusive as to what extent this iodide effect had an autoimmune basis. The mechanisms underlying these phenomena remain unclear but it has been postulated (Roti et al. 1997) that they may involve inhibitory effects of iodine excess on: a) iodide organification, i.e. an inability of some subjects to escape a persistent Wolff-Chaikoff effect, or b) the release of T4 or T3 from the thyroid.

Iodide administration has been also clearly shown to increase the incidence and severity of disease in animals prone to develop spontaneous EAT, such as OS chickens (Bagchi et al. 1985), BB/W rats (Allen et al. 1986) and NOD.H-2^{h4} mice (Rasooly et al. 1996a). Yet,

the effect of iodine on thyroid function remains unclear, since serum thyroid hormones were either not examined (Allen et al. 1986, Bagchi et al. 1985) or found unchanged independent of the accelerated EAT (Rasooly et al. 1996a, Yu et al. 2006b). The enhanced immunogenicity of HI-Tg both at the B- and T- cell level (Champion et al. 1987, Rasooly et al. 1998, Saboori et al. 1998, Sundick et al. 1987) is believed to contribute to pathogenesis with the genetic background of the host playing a pivotal role. In addition, excess iodide administration to strains of mice and rats that do not develop spontaneous EAT does not induce histological changes in the thyroid (Allen et al. 1986, Braley-Mullen and Sharp 1997, Hutchings et al. 1999).

The thyroid is an endocrine organ specialized to concentrate and incorporate available iodide into Tg, forming thyroid hormones – T4 and T3. Serum iodide is actively absorbed from the basolateral membrane into thyrocytes via NIS (Spitzweg and Morris 2002) and translocated into the follicular lumen probably via pendrin - an iodide/chloride transporter present in the apical membrane (Royaux et al. 2000). On the outer site of apical surface, iodide is rapidly oxidated and incorporated into tyrosyl residues along the Tg backbone, which subsequently create T4 and T3 through intramolecular coupling (Carrasco et al. 2000). This organification process is catalyzed by TPO in the presence of an H_2O_2 generator - the thyroid nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (THOXs), also called Dual Oxidase (DUOX), which contain DUOX1 and DUOX2 two genes (De Deken et al. 2000). The pituitary-derived TSH, the main stimulator of synthesis

of thyroid hormones, interacts with the its receptor (TSHR) at the basolateral membrane of the thyrocytes (Carrasco et al. 2000).

Our laboratory has previously reported that HI-Tg is highly immunopathogenic in SJL mice – a strain which does not develop spontaneous thyroiditis – causing EAT of higher incidence and severity as well as stronger B- and T-cell responses than those elicited by normal Tg (Dai et al. 2002). In vitro experiments demonstrated that altered processing of HI-Tg in APC can generate the cryptic pathogenic peptide (2495-2503), and allowed us to postulate that Tg iodination may promote generation of pathogenic epitopes to which immune tolerance has not been previously established. In the present study, we thought to explore a corollary of these findings, i.e. that iodine administration in SJL mice might promote Tg iodination in vivo, and sensitization of autoreactive T cells of the host to p2495 or other pathogenic determinants. Unexpectedly, we observed iodide-induced goiter, hypothyroidism and focal thyroiditis, as well as suppression of Tg-specific immune responses, in these mice.

5.3 RESULTS

High iodine intake induces thyroid goiter and focal thyroiditis in SJL mice

Female mice from the non autoimmune-prone strains SJL and CBA/J mice (8 mice per group) were provided with drinking water supplemented or not with 0.05% NaI. Ten weeks later, all SJL mice placed on NaI developed an approximately 4-5 fold goitrous enlargement in both thyroid lobes- whereas the thyroids of mice fed normal water were unaffected (**Fig. 5.1A**).The majority of the follicles in the goitrous glands were lined with flattened epithelial cells, suggesting metabolic inactivity and hyposecretion of thyroid hormones (**Fig. 5.1B, C, E, F**). In addition, small foci of mononuclear cell infiltration were observed among the thyroid follicles (**Fig. 5.1D & G),** in all SJL mice with high iodine intake with a mean I.I. = 2 (Table 2). These microscopic and histological changes, were not apparent at the 3 wk and 6 wk intervals following the initiation of high iodine intake. In addition, these symptoms were not observed in any of the similarly treated CBA/J mice, suggesting influence of polymorphic genes on the biological response to high iodine intake.

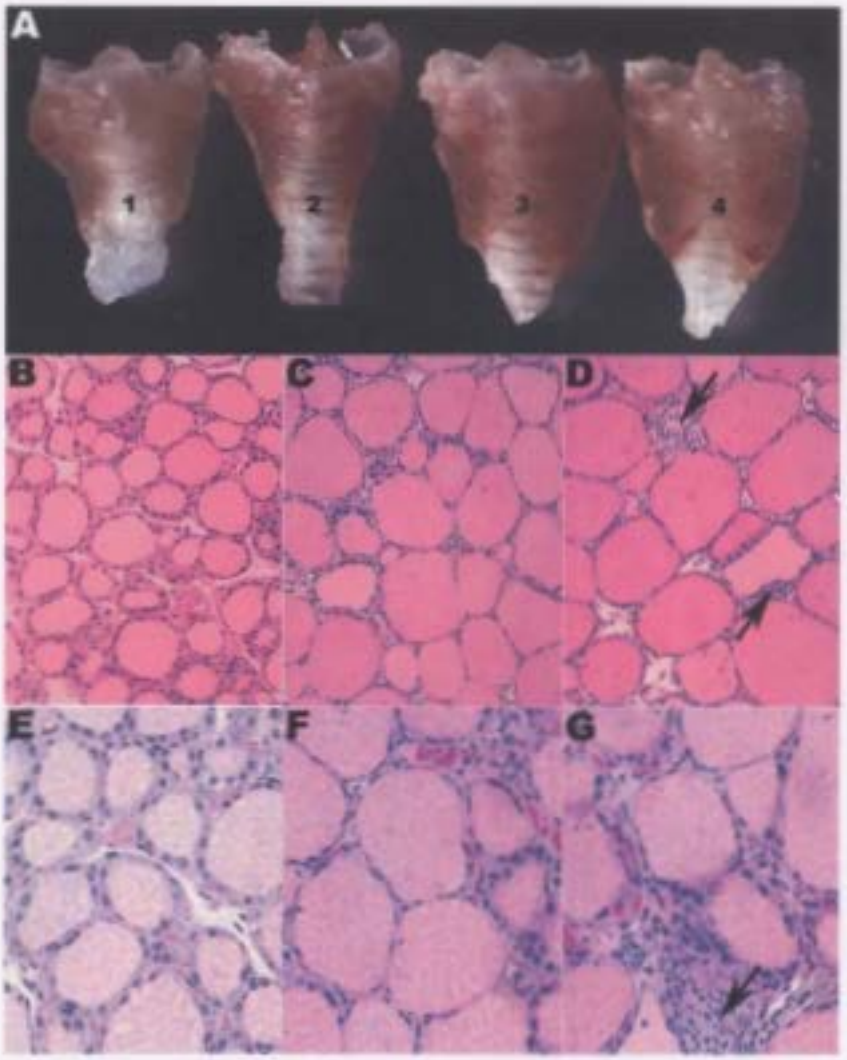

Figure 5.1. Morphological and histological appearance of SJL thyroid glands after 10 wk of high NaI intake.

A, Thyroids from SJL mice fed normal water (# 1 & 2) or water supplemented with 0.05% NaI (# 3 & 4); **B, E,** Histological appearance of normal thyroid follicles; **C, F,** Thyroid follicles of goitrous glands; **D, G,** Focal mononuclear cell infiltrates (arrows) among the follicles of goitrous glands (Magnifications: B, C, D × 200; E, F, G × 400).

High iodine intake does not lead to increased iodide organification and inhibits thyroid hormone release in SJL mice

To determine whether high iodine intake led to enhanced organification of iodide we examined the iodine content in Tg purified from 15 SJL glands after 10 wk of NaI administration. It was found that Tg derived from NaI-treated mice had a lower, although not statistically significant, iodine content than normal Tg (13 vs 20 iodine atoms per monomeric unit) (**Fig. 5.2**). To investigate the effect of iodide on thyroid hormone release, serological determination of TSH and total T4 was performed in 35 SJL mice (7 mice per group per time point) at 1, 3, 6, and 10 wk following administration of NaI or 3 wk after its withdrawal. An equal number of mice were used as controls. As shown in **Fig.5.3A**, the mean total T4 concentration in the experimental group was significantly lower than that of the control group (2.6 vs 3.6 μg/dl, $p<0.0001$), as early as 1 wk following iodide administration. This difference remained significant until 10 wk (1.9 vs 3.0 μg/dl, $p<0.0001$) when goiter formation became evident (**Fig. 5.3A**). A compensatory elevation in mean serum TSH concentration (1.36 vs 0.90, 1.41 vs 1.03, 2.62 vs 0.98, 3.83 vs 104 ng/ml at w1, w3, w6, w10, respectively) was observed in NaI-treated SJL mice vs the controls (**Fig. 5.3B**). Three weeks after NaI withdrawal, the mean total T4 value increased (2.5 μg/dl), approaching that of the control group (2.8 μg/dl), and the mean TSH level decreased but remained significantly higher than that of the controls (218 vs 100 ng/ml) (**Fig 5.3 A&B**). In contrast, hypothyroidism was not observed in CBA/J mice fed NaI at 1, 3 and 6 wk (data not shown) or 10 wk (**Fig. 3 A&B**). These data suggested that high iodine intake exerted a prolonged inhibitory effect not only on

intrathyroidal iodide organification, but also on thyroid hormone release in SJL mice, and that this phenomenon was under the influence of polymorphic genes since it was not observed in CBA/J mice.

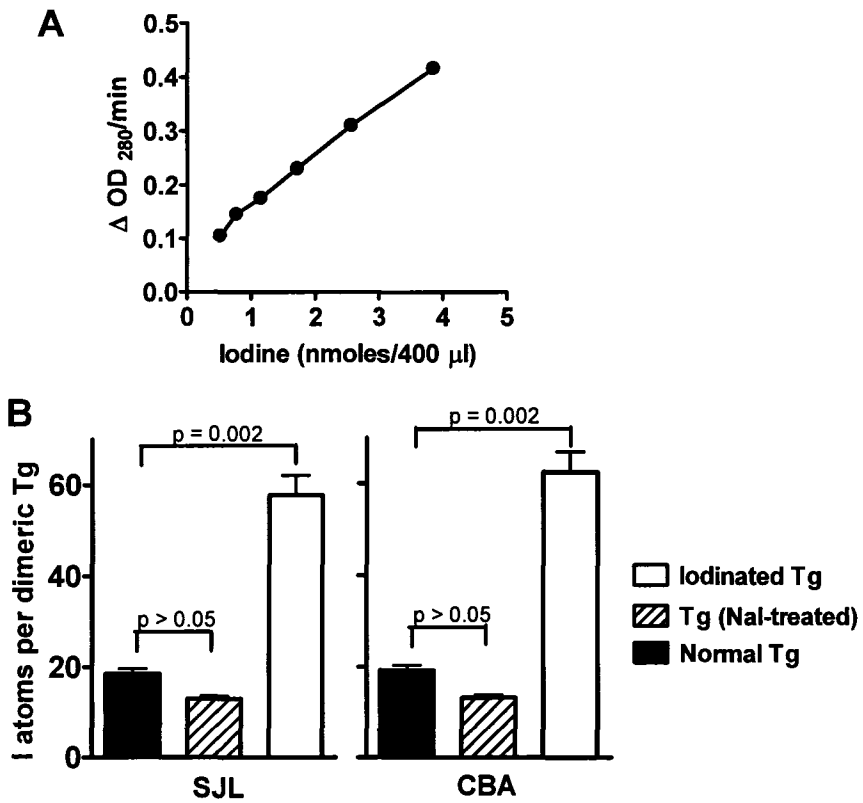

Figure 5.2. Determination of iodine content in Tg preparations.

A, Reduction of absorbance at 410 nm in the first 60 s of the I-catalyzed Ce-As reaction as a function of I added (supplied as T4); **B,** Iodine content in normal Tg (filled bars), Tg purified from 15 goitrous glands 10 wk after the initiation of 0.05% NaI intake (striped bars) or Tg maximally iodinated in vitro by the Iodogen method (open bars), extrapolated from the standard curve. Results are expressed as mean values of two independent assays on the same Tg preparation ± SD. Statistical analysis is performed in Graphpap Phrism using one-way ANOVA.

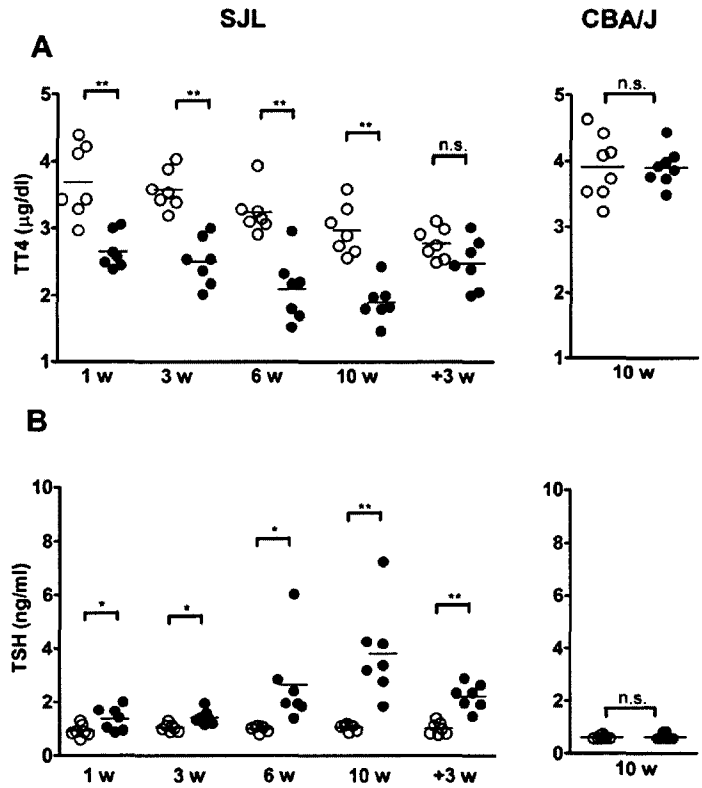

Figure 5.3. Determination of thyroid hormone levels in the sera of SJL and CBA/J mice.

Sera were collected at 1w, 3w, 6w and 10w after placing the animals on normal water (○) or water containing 0.05% NaI (●). After 10 wk of NaI supplementation some mice were placed on normal water for 3 more weeks (+3 w). **A**, Determination of total T4; **B**, Determination of TSH. *, $p<0.05$; **, $p<0.0001$; n.s., not significant.

High iodine intake suppresses Tg-specific responses

In order to assess whether the goitrous hypothyroidism was associated with an immune response to mouse Tg- the major thyroid autoantigen, splenocytes and sera from SJL or CBA/J mice, fed NaI for 10 wk, were tested against Tg, using OVA as a control. Neither T- nor B-cell responses to Tg were detected in these animals (**Table 5.1**). To further examine whether NaI intake predisposed SJL or CBA/J mice to mount an increased immune response to Tg, animals (nine mice per group) were placed on NaI for 10 wk and were subsequently immunized s.c.with 100 μg Tg or OVA in CFA. Nine days later, lymphocytes from draining LN as well as sera, were collected. Surprisingly, Tg-specific LNC or IgG responses were markedly suppressed in NaI-treated SJL as well as CBA/J mice in comparison to control mice placed on normal water **(Fig. 5.4 A,C,E,G)**. This effect was antigen-specific because a similar decreased cellular or humoral response was not observed in mice challenged with OVA (**Fig. 4 B, D, F, H**). Yet, NaI-treated SJL- but not CBA/J - mice developed more severe EAT upon immunization with Tg (data not shown). These results demonstrated that the goitrous hypothyroidism of SJL mice is not associated with an autoimmune response to Tg. Instead, a selective down regulatory effect of high iodine intake on the immune response to Tg was evident in both SJL or CBA/J mice, indicating that this effect does not contribute to the development of hypothyroidism.

Table 5.1 Effects of high iodine intake on induction of thyroiditis and Tg-specific immune responses.

Strain of mouse	Direct induction of thyroiditis [a]								Splenocyte proliferative assay [b] in vitro (S.I.) against		Serum IgG response [c] (405 nm) against	
	Drinking water	Infiltration Index (I.I.)						Mean	Incidence	Tg	OVA	Tg	OVA
		0	1	2	3	4	5						
SJL	*Normal*	8	0	0	0	0	0	0.0 ± 0.0	0/8	0.91 ± 0.14	1.28 ± 0.20	0.091 ± 0.002	0.130 ± 0.003
	NaI	0	1	6	1	0	0	2.0 ± 0.5	8/8*	0.98 ± 0.21	1.23 ± 0.18	0.092 ± 0.001	0.135 ± 0.002
CBA/J	*Normal*	8	0	0	0	0	0	0.0 ±0.0	0/9	1.30 ± 0.32	1.08 ± 0.17	0.120 ± 0.018	0.130 ± 0.014
	NaI	8	0	0	0	0	0	0.0 ± 0.0	0/9	1.82 ± 0.48	1.12 ± 0.24	0.114 ± 0.009	0.132 ± 0.021

[a] SJL and CBA/J mice, treated with 0.05% NaI for 10 weeks, were sacrificed and assessed for thyroiditis induction.

[b] Splenocytes were collected from mice challenged as in (a). S.I. values represent mean ± S.D. values of triplicate wells in the presence of 40 μg/ml of Tg or OVA, respectively. Results are representative of 2-4 independent experiments.

[c] Sera were obtained from mice treated as in (a) and diluted at 1:50 in PBS/Tween/0.1% BSA. ELISA results are expressed as mean OD values of duplicate wells ± SD.

*, $p = 0.0002$.

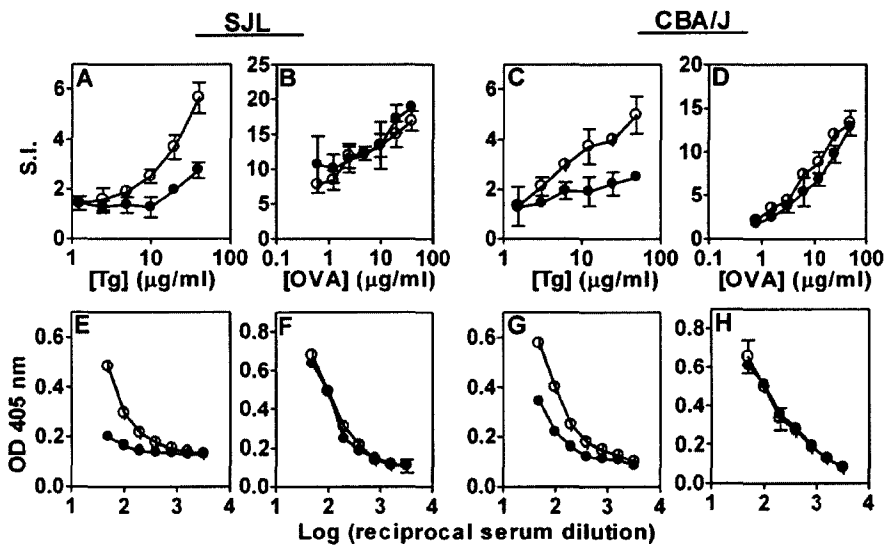

Figure 5.4. Suppressive effects of high iodine intake on Tg-specific humoral and cellular immune responses in SJL and CBA/J mice placed on normal tap water (○) or water containing 0.05% NaI (●) for 10 wk.

A-D, Mice were challenged with Tg or OVA in CFA and nine days later, recall LNC responses to the respective antigen were monitored in vitro. Data represent the mean S.I. values of triplicate wells and are representative of two independent experiments. Background cpm ranged from 2000-5000; responses to the control antigen were undetectable; E-H, serum IgG responses from the mice described above against Tg (E & G) or OVA (F & H).

Intrathyroidal expression of genes involved in iodide transportation and organification

To identify candidate genes that might account for the development of goitrous hypothyroidism, we examined by RT-PCR (30 cycles, listed in **Table 5.2**) the expression levels of thyroidal mRNA encoding Tg, TSHR, NIS, Pendrin, TPO, DUOX1 and DUOX2 at 1 or 10 wk following NaI administration (4 mice per group). It was found that the TSHR mRNA was significantly upregulated at 10 wk in hypothyroid SJL mice but not in similarly treated euthyroid CBA/J mice (**Fig. 5.5 A, B**). At the same time, the high iodine intake downregulated significantly the NIS mRNA expression in CBA/J mice. In contrast, the intrathyroidal expression of NIS mRNA in SJL remained unchanged throughout the duration of the experiment and was not significantly different between mice placed on NaI vs the controls (**Fig. 5.5 A, B**). No significant differences were observed regarding the relative mRNA expression of the other genes in hypothyroid vs euthyroid mice.

Table 5.2. The sequences of PCR primers used in Chapter 5

Gene	Primers		Tm	Product Size (bp)
Tg	Forward	CGGGTACCATGGGGCTTATCAATAG	76	654
	Reverse	CGTCTAGATCATGCATCCTTGGCTC	76	
TSHR	Forward	TGCAACTTGGCCTTTGCAGAT	65.2	520
	Reverse	CCATCCTCTTGGCAATCTTGG	64	
NIS	Forward	TCTTCCTGGCCTGTGCCTACA	65.3	506
	Reverse	GCCCGAGTCCATTCCAGAACT	64.9	
Pendrin	Forward	TCCGAACTCCCGGTGAAAGTGAAT	60	521
	Reverse	TAACAGTAAGCGGATGGCTGCTGA	60	
TPO	Forward	GCACCTTGGATCTGGCATCAC	65.2	502
	Reverse	TGTGGGAAGGTCTCCCTCCAT	65.2	
Duox1	Forward	TGGAGACCTTCTTCAGGCACCTTT	59.9	555
	Reverse	TCTTGATCACTTCCGGCACTTCCA	60	
Duox2	Forward	TATGGCTGCAGTTGTCCTAGCTGT	59.9	305
	Reverse	TAGACACCAGGGTGGGGTCACAGA	60.8	
GAPDH	Forward	CCATCACCATCTTCCAGGAG	62	577
	Reverse	CCTGCTTCACCACCTTCTTG	62	

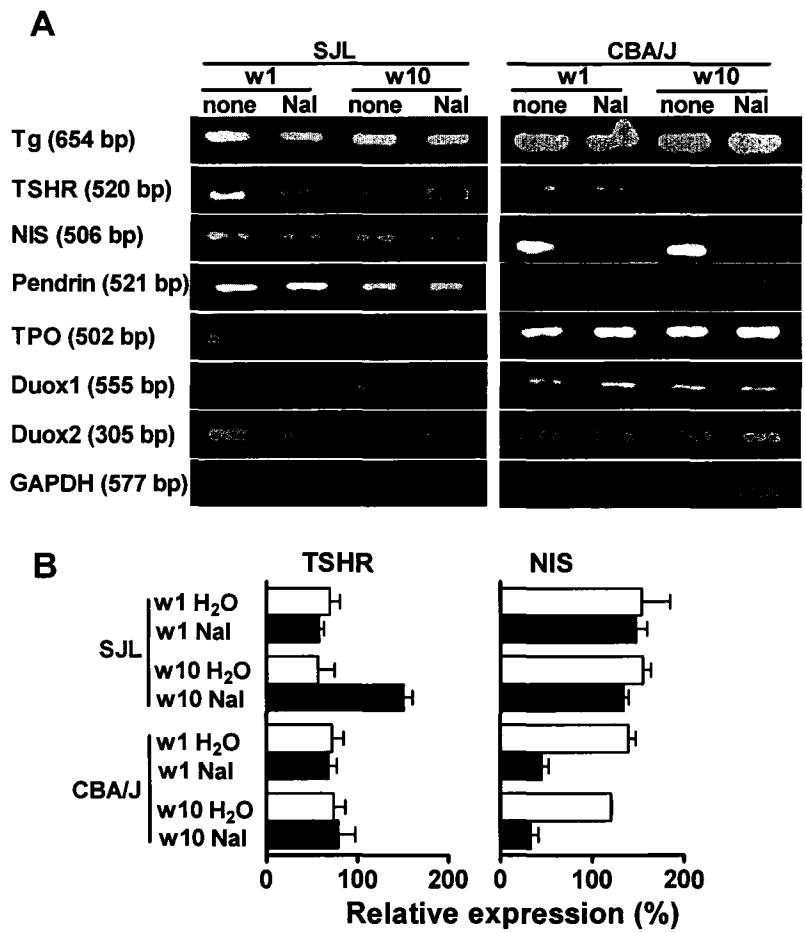

Figure 5.5. Intrathyroidal mRNA expression of candidate genes involved in iodide transportation and organification.

A, RT-PCR analysis of samples obtained from animals (4 mice per group) after 1 and 10 wk of high iodine intake or controls. **B**, Relative expression of mRNA encoding TSHR and NIS in control (open bars) or NaI-treated (filled bars) SJL and CBA/J mice. Relative expression was calculated as the ratio of the optical density of the target gene band to that of the GAPDH band in the same sample and under similar conditions of amplification. The data represent the mean $\pm$ SD of 2 independent experiments.

5.4 DISCUSSION

The current study was initiated to investigate the effects of intrathyrodial formation of HI-Tg, via incorporation of enriched dietary iodine (Sundick et al. 1987), on the development of EAT. Serendipitously, we found both iodide organification and thyroid hormone release were significantly inhibited in SJL, but not CBA/J mice after 10 weeks' NaI administration, leading to the development of goitrous hypothyroidism. Our results are in concordance with previous epidemiological reports in humans (Gao et al. 2004, Khan et al. 1998, Konno et al. 1993, Laurberg et al. 1998, Szabolcs et al. 1997, Teng et al. 2006) and experimental findings in puppies (Castillo et al. 2001), representing the first murine model of iodide-induced hypothyroidism in a non autoimmune-prone strain. The hypothyroidism induced by excessive iodide intake is reversible after iodide withdrawal, confirming earlier studies in humans (Konno et al. 1993, Vagenakis et al. 1973). Our findings also highlight, for the first time, the critical role of genetic factors on iodide metabolism and provide a possible explanation for the prevalence of iodide-induced goiter and/or hypothyroidism in apparently healthy individuals (Gao et al. 2004, Khan et al. 1998, Konno et al. 1993, Laurberg et al. 1998, Szabolcs et al. 1997, Teng et al. 2006).

The mild infiltration in these goitrous glands, together with the suppressed anti-Tg responses, indicate the development of iodide-induced hypothyroidsm is unlikely due to an anti-Tg reactivity (Kimura et al. 2005). The decrease in autoimmune reactivity against Tg was reported previously in Wistar rats placed on 0.05% NaI-supplemented drinking water (Mooij et al. 1994a). The mechanisms underlying this phenomenon are uncler, but

the effects of iodide on synthesis, release and antigen processing of Tg (Rasmussen et al. 2002, Dai et al. 2002, Fouchier et al. 1983) may interfere the homeostasis of Tg-specific autoreactive T cells and/or Treg. Of note, we need to keep in mind that autoimmune responses against other thyroid A-Ags may develop following iodide administration, which have not been tested in the current study. It was reported in NOD- $H2^{h4}$ that spenic T cell responses to TPO were detected three weeks after iodide administration, but no anti-TPO Abs were found at this or later time points (Allen et al. 1987). We do not know in the current murine model, however, whether goitrous hypothyroidism and EAT occur independently or synergistically. In other models, excessive iodide administration has been shown to induce goiter and hypothyroidism in hemithyroidectomized rats regardless of EAT development (Allen et al. 1986), or to strikingly increase the incidence of EAT in BB/W rats in the absence of hypothyroidism (Wolff and Chaikoff 1948, Wolff et al. 1949). These findings argue that iodide-induced hypothyroidism occurs on an autoimmune basis.

Large amounts of iodide have been shown to inhibit thyroid hormone biosynthesis (the "Wolff-Chaikoff effect") (Wolff et al. 1949) and its release (Vagenakis et al. 1973), caused by high levels of intrathyrodial iodide. Normal subjects, such as CBA/J mice herein, exposed to chronic iodide excess, do not develop hypothyroidism due to so-called "adaptation" or "escape" phenomenon (Eng et al. 1999, Eng et al. 2001, Ferreira et al. 2005, Spitzweg et al. 1999b, Uyttersprot et al. 1997). It was suggested that this "escape" is mediated by a significant decrease in NIS expression following iodide administration

(Belshaw and Becker 1973, Mahmoud et al. 1986). In this study, we observed a significant reduction in intrathyrodal NIS mRNA mice following iodide administration in CBA/J, but not SJL mice. Expression of pendrin, another iodide transporter in the apical membrane of thyrocytes, did not differ before and after iodide administration in both SJL and CBA/J mice, which are in agreement with in vitro findings in a rat thyrocyte line FRTL-5 (Royaux et al. 2000). The unaffected NIS expression in SJL mice at the mRNA level, and possiblely at the protein level, may facilitate the uptake of iodide in the basolateral membrane of thyrocytes and increase intrathyroidal iodide concentrations in the presense of high iodide intake, leading to a prolonged "Wolff-Chaikoff effect". The elevated intrathyroidal iodide will probably continue exert their inhibitory effects on iodide organification to generate lowly iodinated Tg, as seen in SJL mice herein (**Fig. 5.2**), and lead to the decrease in thyroid hormone synthesis and release (**Fig.5.3**). Interestingly, serum total T4, but not TSH, appeared to decrease with age in SJL mice placed on normal water, and this phenomenon has not been reported previously. However, serum total T4 in approximately 18w-old CBA/J mice was maintained at similar levels as in 9w-old SJL mice. It is unknown whether there exist age-related thyroid abnormalities in SJL mice, which may predispose the animals to iodide-induced hypothyroidism.

The highly elevated intrathyroidal iodide level may interferes with thyrocyte growth and induces thyrocyte necrosis in situ due to its toxic effects (Gallucci and Matzinger 2001). These injured thyrocytes may lose their capability of thyroid hormone synthesis and

release, causing the development of goitrous hypothyroidism. Furthermore, disruption of cellular intergrity during cell necrosis releases many kinds of inflammatory stimuli, referred to "danger signals" (Drakesmith et al. 2000, Ludewig et al. 1999, Turley 2002), which may recruit the mononuclear cells into thyroid gland, leading to EAT. The presence of infiltrating cells may further accelerate the disfunction of thyroid follicles. Further studies are needed to define the polymorphic genes that confer SJL mice susceptibility to iodide-induced hypothyroidism and to determine the mechanisms underlying this phenomenon.

CHAPTER 6

MATURATION OF DENDRITIC CELLS BY NECROTIC THYROCYTES FACILITATES INDUCTION OF EXPERIMENTAL AUTOIMMUNE THYROIDITIS

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Haiyan S. Li, Panayotis Verginis and George Carayanniotis

Divisions of Endocrinology and Basic Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

6.1 ABSTRACT

DC maturation is required for efficient presentation of autoantigens leading to autoimmunity. In this report, we have examined whether release of tissue antigens from necrotic thyroid epithelial cells can trigger DC maturation and initiation of a primary antiself response. DC were co-cultured with either viable (VT/DC) or necrotic (NT/DC) thyrocytes, and their phenotypic and functional maturation as well as immunopathogenic potential were assessed. Significant up-regulation of surface MHC class II and costimulatory molecule expression was observed in NT/DC but not in VT/DC. This was correlated with a functional maturation of NT/DC, determined by IL-12 secretion. Challenge of CBA/J mice with NT/DC – but not with VT/DC - elicited Tg-specific IgG as well as Tg-specific $CD4^+$ T-cell responses and led to development of experimental autoimmune thyroiditis. These results support the view that thyroid epithelial cell necrosis may cause autoimmune thyroiditis via maturation of intrathyroidal DC.

6.2 INTRODUCTION

DC, loaded with self proteins or peptides, are to provoke organ-specific autoimmune diseases (Knight et al. 1988). This was first observed in EAT with Tg-pulsed DC (Watanabe et al. 1999) and was later confirmed in the same (Dittel et al. 1999, Weir et al. 2002) or other animal models (Turley 2002). These observations, the unrivalled capacity of DC to activate naïve T cells, and the detection of DC in lesions associated with numerous autoimmune diseases (Canning et al. 2003, Many et al. 1995, Hala et al. 1996, Voorby et al. 1990), including thyroiditis (Hawiger et al. 2001, Turley 2002, Veeraswamy et al. 2003, Lutz and Schuler 2002), have strongly argued for DC involvement in the initiation of autoimmunity. The maturation stage of DC seems to play a pivotal role in this process: under homeostatic conditions, immature DC are believed to continually transport autoantigens to draining lymph nodes, process and present them to cognate T cells in a sub-stimulatory context, leading to T-cell tolerance (Gallucci and Matzinger 2001). Under the influence of endogenous "danger signals" released by tissues undergoing stress, damage or abnormal death, or exogenous danger signals elaborated by pathogens, DC undergo maturation (Gallucci et al. 1999, Guermonprez et al. 2002, Sauter et al. 2000). During this terminal differentiation stage, DC generate high levels of peptide-MHC class II complexes on their surface and up-regulate costimulatory molecule expression, emerging as ideal APC for naïve T-cells.

Necrotic cells, i.e. disintegrated cells which have released their cell contents prior to their ingestion by phagocytes, can cause mouse and human DC maturation in vitro (Savill et al. 2002). However, the implications of these findings in the induction of organ-specific autoimmunity have not been adequately explored. Primary necrosis is triggered by noxious stimuli such as toxins, hypoxia and extremes of temperature (Li and Boyages 1994, Bagchi et al. 1995, Ruwhof and Drexhage 2001a), whereas secondary necrosis refers to the eventual disintegration of cells that have initially undergone apoptosis but have not been captured by phagocytes. To the extent that chronic dietary iodine excess is known to have toxic effects on thyrocytes of animals prone to autoimmunity (Verginis et al. 2005, Macatonia et al. 1995), we sought in this study to test: a) whether necrotic primary thyrocytes can mediate maturation of DC; and b) whether DC that have ingested syngeneic necrotic thyrocytes can elicit EAT or thyroid antigen-specific responses, following their adoptive transfer in CBA/J hosts. LPS-matured DC loaded with Tg, the major thyroid antigen, were used as controls in the study to ensure the efficiency of T cell priming by DC.

6.3 RESULTS

Generation of Tg-secreting thyrocytes

Primary thyrocytes were freshly prepared from CBA/J mouse thyroids, and cultured in 8-well chamber slide in complete F-12 medium. After one week, thyrocytes were washed in PBS and treated with rabbit anti-Tg antibodies (primary antibody) and FITC-labeled goat-anti-rabbit antibodies (secondary antibody). Thyrocytes directly labeled with secondary antibodies were used as control. As shown in **Fig. 6.1**, almost 100% of the cultured cells showed Tg labeling in their cytoplasm, confirming their thyrocyte origin.

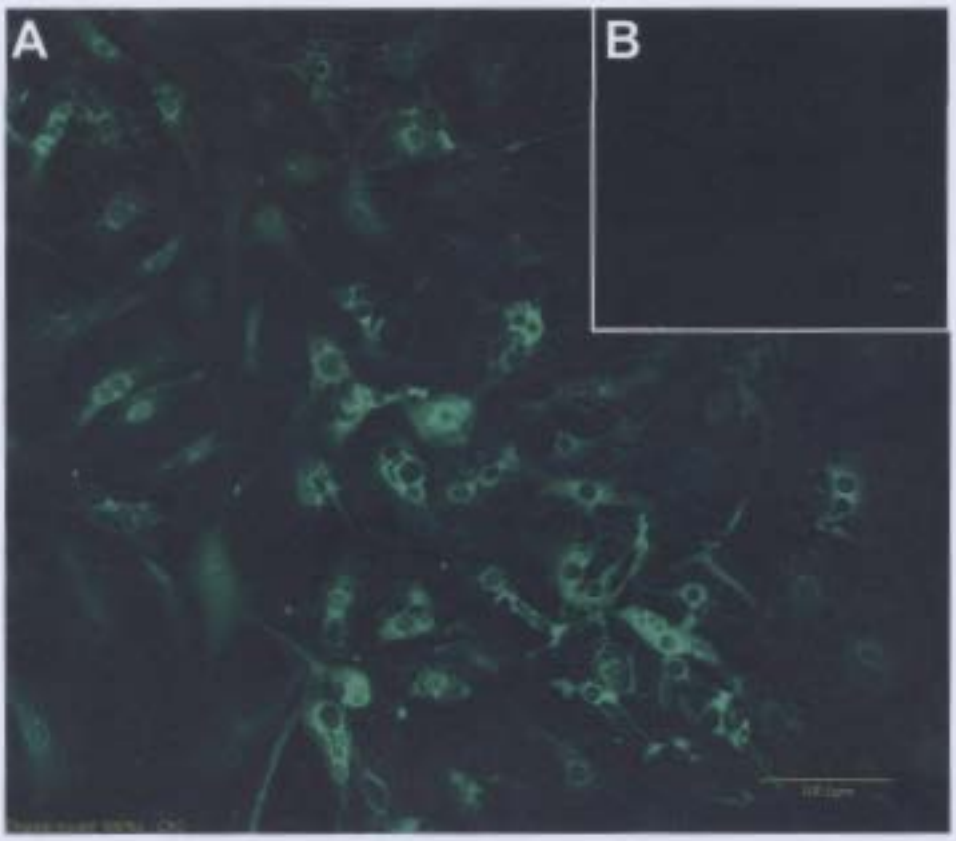

Figure 6.1. Immunofluorescent labeling of intracellular Tg in primary thyrocytes.

A, Primary thyrocytes were isolated from CBA/J mice, cultured for 7 days, and stained with rabbit anti-Tg antibody followed by FITC-goat anti-rabbit antibodies. **B**, Control thyrocytes were stained with second antibody only.

Necrotic thyrocytes stimulate DC maturation

Necrotic thyrocytes were induced by 4 cycles of freeze- thaw as described in Chapter 2. To test whether exposure to necrotic thyrocytes triggers DC maturation, phenotypic markers of DC, co-cultured with necrotic (NT/DC) or viable thyrocytes (VT/DC) for 24 hours, were assessed by flow cytometry. DC treated with LPS (LPS/DC) or untreated DC were used as controls. The expression of MHC class II, CD80, CD86, and CD40 markers in VT/DC, (70%, 70%, 31% and 4%, respectively), was similar to untreated DC (**Fig 6.2A**). In contrast, these markers were significantly up-regulated in NT/DC (87%, 88%, 48% and 9%, respectively) (**Fig 6.2A**), as well as in LPS/DC with the exception of CD86 and CD40 which were higher in LPS/DC (68% and 29%, respectively). IL-12 secretion, a critical parameter of DC functional maturation (Ma et al. 2005), was also monitored by sandwich ELISA in culture supernatants. As shown in **Fig 6.2B**, necrotic, but not viable, thyrocytes activated DC to release significantly higher amounts of IL-12 (approximately 400 pg/ml) than those found in cultures of untreated DC (< 20 pg/ml). These data clearly demonstrated that exposure to necrotic thyrocytes can trigger both phenotypic and functional DC maturation.

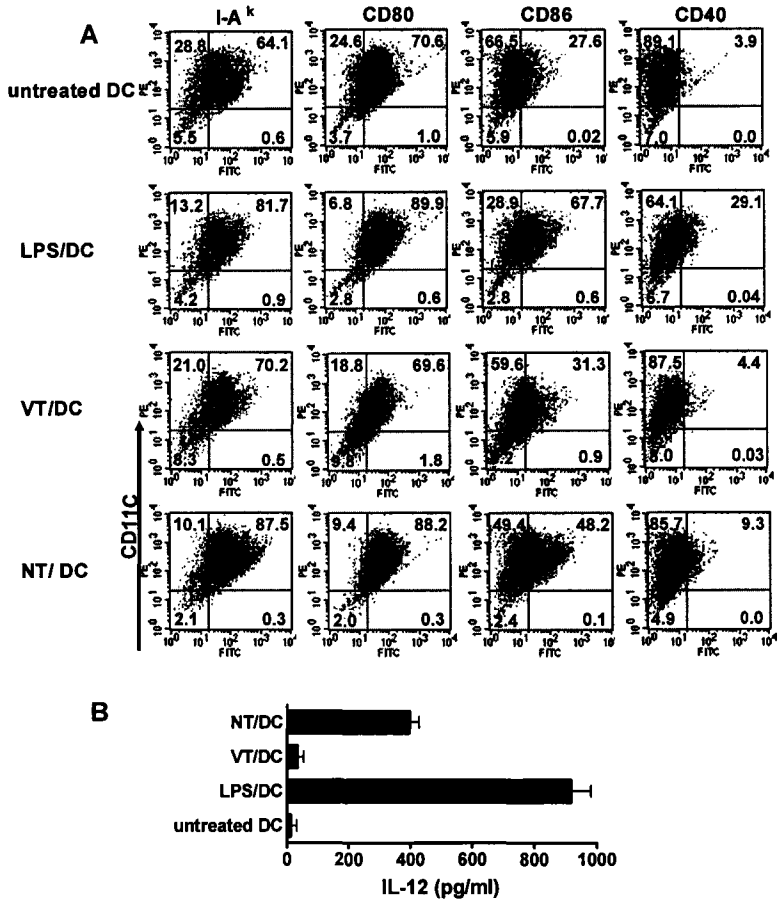

Figure 6.2. Phenotypic and functional analysis of DC.

A, Phenotypic analysis of DC after exposure to various stimuli by double labeling with PE-anti-CD11c mAb, and FITC-conjugated mAbs against MHC class II, CD80, CD86 or CD40. **B**, Functional analysis of DC exposed to the same stimuli as in (**A**), as determined by IL-12 secretion in the culture supernatants. The results are expressed as means $\pm$ S.D. of triplicate wells. Data are representative of two independent experiments in (**A**) and (**B**).

DC exposed to necrotic thyrocytes elicit Tg-specific T- and B-cell responses.

To test the immunogenic potential of DC exposed to necrotic or viable thyrocytes, CBA/J mice (8 mice per group) were i.p. challenged twice (on d1, d15) with 2 x 10^6 NT/DC or VT/DC. Additional groups of mice received the same number of Tg- or OVA- loaded, LPS-matured DC (Tg/DC, OVA/DC). It was observed that splenic $CD4^+$ T cells exhibited low but significant proliferative responses to Tg following priming with Tg/DC or NT/DC (S.I. = 3.3 $\pm$ 0.4 or 3.2 $\pm$ 0.6, respectively) (**Fig 6.3A**). In contrast, Tg-specific proliferative responses were undetectable in mice challenged with OVA/DC or VT/DC. Significant amounts of IL-2 and IFN-γ were detected in culture supernatants of Tg/DC- and NT/DC-activated $CD4^+$ T cells (**Fig 6.3 B,C**), whereas IL-4 or IL-10 were undetectable (data not shown). As expected, challenge with OVA/DC elicited strong OVA-specific proliferative T-cell responses and IL-2 or IFN-γ release. Interestingly, mice challenged with NT/DC mounted strong Tg-specific IgG responses which were significantly higher than those detected in the sera of Tg/DC-challenged mice (Mean OD 405 nm = 1.195 vs 0.548, $p<0.001$) (**Table 6.1**). These results demonstrated that NT/DC were strongly immunogenic, as they could initiate both B- and T-cell responses against Tg, the most abundant thyroid antigen.

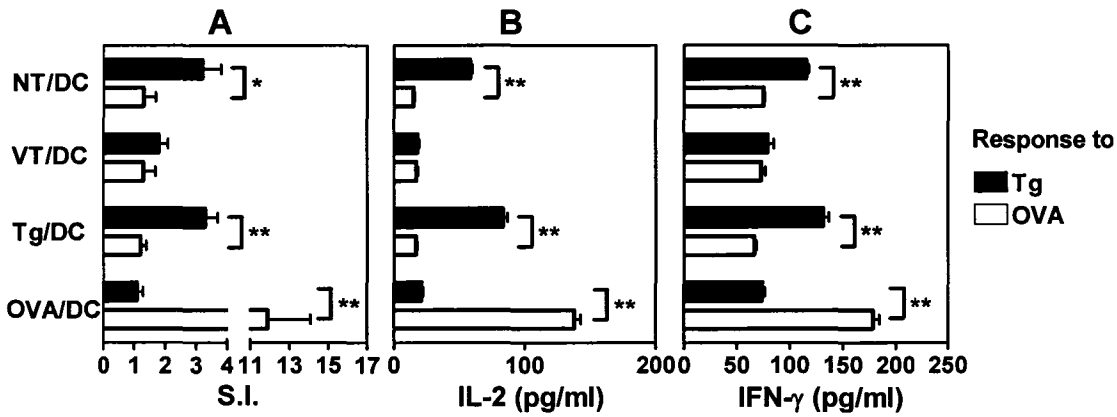

Figure 6.3. NT/DC induce Tg-specific Th1 responses.

A, On d1 and d15, CBA/J mice received an i.p.injection of 2×10^6 DC exposed to the stimuli shown. Two weeks after the last challenge, purified splenic $CD4^+$ T cells were co-cultured with syngeneic mitomycin C-treated DC (APC) to test recall proliferative responses to Tg or OVA. Data represent the mean S.I. values of triplicate wells, obtained at antigen concentration of 100 μg/ml. Background cpm ranged from 1600 to 2200. **B,C**, Cytokine determination by sandwich ELISA in 48-h supernatants of the corresponding cultures is shown in (**A**). Results are representative of two separate experiments.

Table 6.1. EAT and IgG responses induced by DC exposed to various stimuli

	Induction of EAT						Serum IgG response (OD 405 nm) against	
	Infiltration Index (I.I.)					# of mice with EAT		
	0	1	2	3	Mean ± SD		Tg	OVA
NT/DC	4	2	1	1	0.88 ± 1.13	4/8	1.195 ± 0.010	0.193 ± 0.006
VT/DC	8	0	0	0	0.00	0/8	0.285 ± 0.021	0.176 ± 0.005
Tg/DC	3	1	1	3	1.50 ± 1.41	5/8	0.548 ± 0.001	0.197 ± 0.006
OVA/DC	8	0	0	0	0.00	0/8	0.192 ± 0.004	1.615 ± 0.010

CBA/J mice (8 mice per group) were i.p. challenged with 2×10^6 of the indicated DC and boosted, 2 weeks later, with the same number of DC. Thyroids were removed 28 days after the initial challenge for EAT assessment. At the same time, immune sera were collected, pooled and diluted at 1:30 in PBS/Tween/0.1% BSA for IgG determination. ELISA results are expressed as mean OD values of duplicate wells ± SD.

DC exposed to necrotic thyrocytes mediate EAT development

To assess the pathogenicity of NT/DC, the thyroids of CBA/J mice (8 mice per group) treated as described above, were obtained on day 28 for histological assessment. As shown in **Table 6.1**, four out of eight mice receiving NT/DC developed EAT (mean I.I.= 0.9 $\pm$ 1.1) with thyroid infiltration indices ranging from 1-3 **(Fig. 6.4)**. The EAT incidence was similar to that of the group challenged with Tg/DC, since five out of eight mice presented with thyroiditis, albeit of higher severity (mean I.I.= 1.5 $\pm$ 1.4). Mononuclear cell infiltration of the thyroid was undetectable in mice challenged with either VT/DC or OVA/DC. These data directly support the notion that DC which engulf necrotic thyroid epithelial cells can initiate an autoimmune response leading to development of thyroiditis.

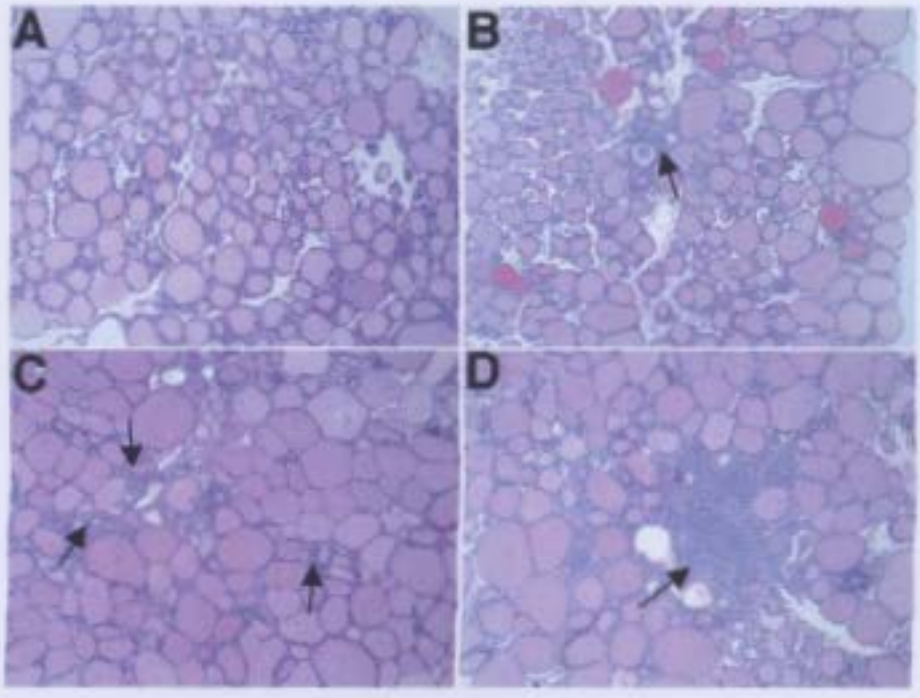

Figure 6.4. Histological appearance of EAT elicited by NT/DC.

On d1 and d15, CBA/J mice were i.p. challenged with 2×10^6 indicated NT/DC. Two weeks after boosting, the mice were sacrificed and the thyroid glands were removed for EAT assessment. (**A**) Normal gland, I.I. = 0; (**B**) Interstitial accumulation of inflammatory cells (arrows), I.I. = 1; (**C**) One or two foci of inflammatory cells (arrows), I.I. = 2; (**D**) Diffuse infiltration, 10–40% of total area (arrows), I.I.= 3. Magnification: × 100.

6.4 DISCUSSION

Our present findings show that DC which have captured necrotic thyrocytes can undergo maturation, and enable the immunogenic presentation of thyroid antigen(s) – such as Tg - on their surface, leading to development of EAT. To our knowledge, this is the first report showing that DC can precipitate an organ-specific autoimmune disease following ingestion of necrotic epithelial tissue. Analogous findings have been recently reported for a model of a systemic autoimmune disease in which DC ingesting necrotic splenocytes induced strong anti-dsDNA antibody responses and accelerated disease progression in lupus-prone mice (Golstein and Dumont 1996, Many et al. 1992). Excess iodide is well known to bring about thyrocyte necrosis both in vitro (Bagchi et al. 1995) and in experimental animals prone to thyroiditis such as OS chickens (Li and Boyages 1994), BB/W rats (Many et al. 1995) and NOD mice (Basaria and Cooper 2005). In humans, administration of the potent anti-arrhythmic drug amiodarone, which is very rich in iodine, has been linked to the pathogenesis of thyroiditis, possibly through the release of autoantigens from the injured thyroid (Arscott and Baker, Jr. 1998). Factors that promote enhanced apoptosis of thyrocytes (Ip and Lau 2004) may also contribute in these processes since apoptotic cells may undergo secondary necrosis if not removed in time (Ishii et al. 2001).

The maturation stimuli released by the disintegrated necrotic thyrocytes are unknown but may include genomic DNA (Basu et al. 2000), heat shock proteins (Rovere-Querini et al.

2004), the high-mobility group B1 protein (Shi et al. 2003) or uric acid (Canning et al. 2003). Under the conditions described herein (i.e. culturing of necrotic thyrocytes with DC at a 10:1 ratio), it is evident that enough antigenic material can be released to induce Tg-specific Th1 responses which were similar in magnitude to those obtained by DC loaded with purified Tg. Intrathyroidal DC are prime candidates for antigen capture in vivo and a small increase in the number of DC, clustering in the thyroidal interstitium, is one of the first signs of developing autoimmunity (Molne et al. 1994). Thyroid tissue, obtained predominantly from patients with Graves' disease, shows the presence of perifollicular immature DC at the basal surface of thyrocytes, with long cytoplasmic protrusions which penetrate the tight junctions between adjacent thyrocytes (Quadbeck et al. 2002). In addition, mature DC have been observed within lymphoid - like clusters in close proximity to $CD4^+$ T cells (Sallusto and Lanzavecchia 1999) in agreement with the concept that mature DC that fail to migrate to lymph nodes may serve as nucleation sites for chronic inflammatory reaction (Knight et al. 1988, Watanabe et al. 1999). According to current theory, however, DC ingesting necrotic thyrocytes are expected to reach the draining lymph nodes and activate thyroid antigen-specific naïve T cells there.

Tg/DC induced EAT with similar incidence but higher severity than that elicited by NT/DC. These findings confirm earlier observations that Tg-pulsed DC can initiate EAT (Wykes et al. 1998). It is quite likely, however, that NT/DC display on their cell surface T-cell epitopes of other thyroid antigens such as thyroid peroxidase; but the extent of other antigen participation, or the involvement of antigenic competition in this process,

remains unknown. The capacity of Tg/DC or NT/DC to elicit significant Tg-specific IgG responses is also in agreement with the finding that DC can interact directly with naïve B cells to transfer unprocessed antigen and initiate class switching (Casiano et al. 1998, Rodenburg et al. 2000). It is not clear why NT/DC induce stronger anti- Tg IgG responses than Tg/DC since maturation of DC was mediated by distinct stimuli, in each case. In addition, it is not known whether Tg freshly released from necrotic cells may have minor conformational differences from lyophilized Tg preparations which could be detected by B cells. Cleavage of autoantigens during necrotic cell death seems to differ from the caspase-dependent proteolysis observed in apoptosis (Rodenburg et al. 2000). An intriguing possibility is that Tg processing in NT/DC may take place in a different manner than in Tg/DC, revealing the generation of cryptic determinants to which tolerance has not been established (Carayanniotis 2003). This is a testable hypothesis given the large number of pathogenic but cryptic Tg determinants already mapped (Dai et al. 2002). A similar hypothesis can be made in iodide-induced thyrocyte necrosis since we have observed that the processing of highly iodinated Tg can promote the generation of a cryptic pathogenic determinant (Anderson and Kuchroo 2003, Kyewski et al. 2002). Lastly, it will be interesting to test whether use of Tg- pulsed tolerogenic DC may prevent EAT induction by NT/DC, as has been recently observed for EAT elicited by Tg in adjuvant, or whether apoptotic thyrocytes may be needed to generate tolerogenic DC for this purpose.

CHAPTER 7

DETECTION OF THYROGLOBULIN MRNA AS TRUNCATED ISOFORM(S) IN MOUSE THYMUS

(This work was published in *Immunology 2005 May; 115 (1): 85-9.* This is a modified version)

Haiyan S. Li and George Carayanniotis

Division of Endocrinology, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

7.1 ABSTRACT

Recent studies employing RT-PCR have demonstrated the intrathymic presence of mRNA for various autoantigens, including Tg. Deliberations on mechanisms of central tolerance usually assume that this approach detects intact mRNA transcripts that can be translated to express the whole autoantigen in the thymus. In this study, we have tested this assumption with mRNA transcripts of mouse Tg which encode at least thirteen pathogenic peptides, scattered over a large (8.5 kb) sequence. We found that mRNA encoding eleven out of these thirteen Tg peptides was present in both the thyroid and the thymus of CBA/J mice with no apparent temporal fluctuations in expression from birth to 12 weeks of age. Interestingly, detection of these sequences was also demonstrable in liver and kidney, but not in muscle. However, mRNA encoding two pathogenic peptides, (a.a. 1-12) and (a.a. 1579-91), was detected intrathyroidally but not in the other tissues. Further analysis by RT-PCR showed that Tg mRNA transcripts in thymus, liver and kidney lack the 1-915 bp and 961-5013 bp segments, spanning exons 1-7 and 9-22, respectively. These data strongly suggest that certain known and perhaps other as yet unmapped pathogenic T-cell epitopes of Tg cannot be encoded by the truncated isoform(s) of intrathymic Tg mRNA. These findings also imply that central tolerance to endogenous Tg produced by thymic epithelial cells may be incomplete.

7.2 INTRODUCTION

The physiological expression of peripheral tissue-specific antigens in the thymus has been well documented in recent years as reviewed in (Derbinski et al. 2001, Kyewski et al. 2002). This phenomenon of "promiscuous gene expression" (Egwuagu et al. 1997, Liu et al. 2001a, Pugliese et al. 1997, Vafiadis et al. 1997) has been considered to play a pivotal role in the shaping of the autoreactive T-cell repertoire, since the levels of intrathymic expression of several tissue antigens inversely correlate with susceptibility to organ-specific autoimmunity (Targoni and Lehmann 1998). Deletion of high affinity autoreactive T cells by the self antigen expressed in the thymus is one mechanism that could account for these observations (Nave et al. 1987). In some instances, however, self-reactive cells may escape thymic deletion because the isoform of the antigen expressed in the thymus differs from that expressed in the target organ. This has been best illustrated in studies of EAE and the target antigen PLP, the main protein of the myelin sheath. Intrathymic PLP mRNA exists predominantly as a splice variant, DM20, which lacks a specific loop of 35 amino acids (Anderson et al. 2000, Klein et al. 2000). It has been shown that T cell tolerance to PLP is restricted to those epitopes included in DM20 (Carayanniotis 2003, Gentile et al. 2004).

Tg is the largest autoantigen known –with a molecular mass of 660 kDa in its homodimeric form - and the most abundant glycoprotein of the thyroid gland. Mapping studies have so far identified thirteen Tg peptides encompassing T-cell epitopes which elicit EAT when administered with adjuvant into mice (Carayanniotis and Kong 2000,

Carayanniotis 2003). However, none of these epitopes has been classified as dominant by proliferative assays in vitro, and the conditions that might promote their participation in the disease process remain speculative (Heath et al. 1998). Several studies using RT-PCR and other detection methods have demonstrated promiscuous intrathymic Tg gene expression in rats (Derbinski et al. 2001), mice (Spitzweg et al. 1999a) and humans (Mor et al. 1998). In contrast, Mor et al. (Carayanniotis 2003) could not find evidence for intrathymic expression of Tg in rats using RT-PCR and primers that amplified the 532-832 bp fragment. These apparently contrasting results, the large size of the Tg molecule (2748 aa), and the expression of autoantigen isoforms in the thymus in other animal models, prompted us to examine whether intrathymic Tg gene detection by RT-PCR could be significantly influenced by the choice of the gene region used for primer design. In this effort, we used thymi of CBA/J mice from birth to 12 weeks of age and the cDNA sequences encoding the known pathogenic Tg peptides as landmarks, since they are scattered over the whole length of the Tg molecule. Tg gene detection in other extrathyroidal tissues such as liver, kidney and muscle was also investigated by the same approach.

7.3 RESULTS

7.3.1 mRNA encoding 2 out of 13 pathogenic Tg peptides is undetectable in the thymus

The expression of mRNA encoding each known pathogenic peptide of Tg was first determined by RT-PCR in the thyroid, thymus and muscle tissues of 12 wk-old CBA/J mice (Primers are listed in **Table 7.1**). As expected, mRNA encoding all thirteen Tg peptides was abundantly expressed in the thyroid. However, the mRNA transcripts for 2 peptides, p1 (a.a. 1-12) and p1579 (a.a. 1579-91) were undetectable in the thymus **(Fig. 7.1)**. An identical mRNA expression profile for all Tg peptides under investigation was obtained with thyroid and thymus tissues from newborn, 2-, 6-, and 12- wk old CBA/J mice **(Fig. 7.2A&B)**, suggesting that these data did not reflect any temporal influences on expression. None of the 13 mRNA transcripts was detected in muscle **(Fig. 7.1)** and this was also confirmed at all the above time points tested.

Table 7.1 The sequences of PCR primers used in Chapter 7.

Peptide/ fragment	a.a. coordinates	b.p. coordinates	Forward primer*	Reverse primer*	Product Size (bp)
p1	1-12	1-36	AGGGTACCAACATCTTTGAG	TGTCTAGAGGGGCGGAGTGG	58
p306	306-320	916-960	AGGGTACCGATGGTCACTACCAA	TGTCTAGACTGGGCATCCACACA	79
p1579	1579-1591	4735-4773	AGGGTACCGACTCCCCGCTGGTG	TGTCTAGAGAAGCTGCAGGCCTC	72
p1672	1672-1711	5014-5133	AGGGTACCCAGAAGAGCTTCGAA	TGTCTAGAACAGCAGGAATCATT	154
p1826	1826-1836	5476--5508	AGGGTACCGACTTTCCAGGAGAT	TGTCTAGAGGTAATGTCCACAGG	67
p2102	2102-2116	6304-6348	AGGGTACCAGTAACTTCTCCATG	TGTCTAGAAAGGCAGTCCTGGTG	79
p2340	2340-2359	7018-7077	AGGGTACCCTGCTGGACCAAGTG	TGTCTAGATGTCACACGCTGAGG	94
p2494	2494-2510	7483-7533	CGGGTACCATGGGGCTTATCAATAG	CGTCTAGATCAGCCTTGGCTCTCTT	73
p2549	2549-2560	7645-7680	AGGGTACCTTGGAGCACTCCACA	TGTCTAGAGGCATTCTCCAGTGC	70
p2596	2596-2608	7786-7824	AGGGTACCCCCGAAAGCTATGGC	TGTCTAGAAAAAGCATATTGAAC	73
p2695	2695-2713	8083-8136	CGGGTACCATGTGCTCCTTCTGGT	CGTCTAGATCATGCATCCTTGGCTC	82
p2730	2730-2743	8188-8229	AGGGTACCGTTGGACCTGGATTA	TGTCTAGATTTGCTGTAGCTCTT	82
A	306-520	916-1560	AGGGTACCAACATCTTTGAG	TGTCTAGACTTCTCAGACACACG	645
B	516-745	1546-2235	ATGGTACCCGTGTGTCTGAGAAG	TGTCTAGAAGAGGCACTGCACTGAG	690
C	741-980	2221-2940	AGGGTACCCCTCAGTGCAGTGCC	TGTCTAGACTGAGCAGCCAAGCG	720
D	976-1225	2926-3675	AGGGTACCCGCTTGGCTGCTCAG	TGTCTAGACTGCTGAACAGTCGT	750
E	1221-1435	3661-4305	AGGGTACCACGACTGTTCAGCAG	TGTCTAGAAGCATCCTGTCTGGT	645
F	1436-1711	4306-5133	AGGGTACCCTGGGCTGTGTGAAA	TGTCTAGAACAGCAGGAATCAT	828
β-actin	--	--	GCTCTTTTCCAGCCTTCCTT	CTTCTGCATCCTGTCAGCAA	177

*All primers are given in 5' to 3' directions; underlined sequence is *KpnI* site in forward and *XbaI* site in reverse primers

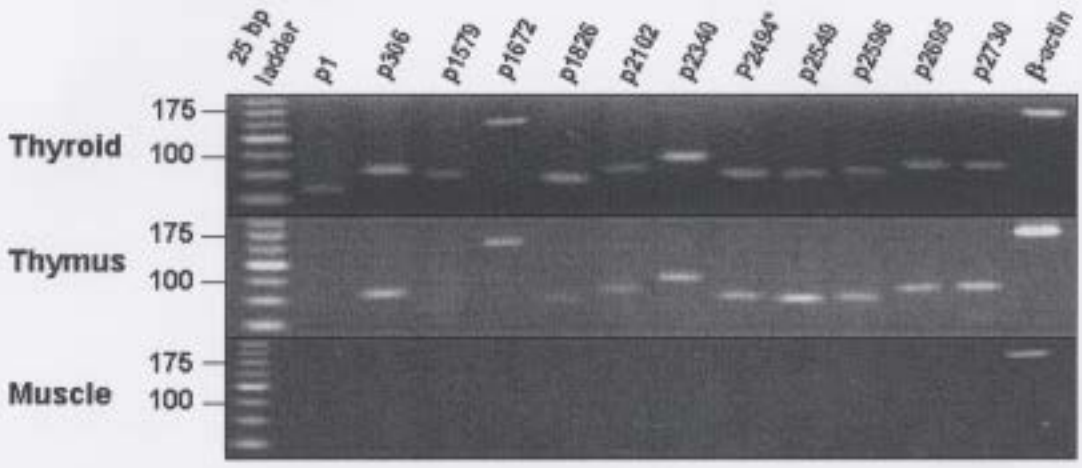

Figure 7.1. mRNA sequences encoding two pathogenic Tg peptides (p1 and p1579) are detectable in the thyroid but not in the thymus of CBA/J mice.

Data are representative of individual samples from 5 mice, as detected by RT-PCR with 35 cycles. mRNA from muscle tissue was used as a control. * The expression of mRNA encoding the thirteen known pathogenic Tg peptides was assessed in 12 tracks since the p2494 mRNA sequence codes for two overlapping pathogenic peptides (2495-2503) and (2498-2506) (Heath et al. 1998).

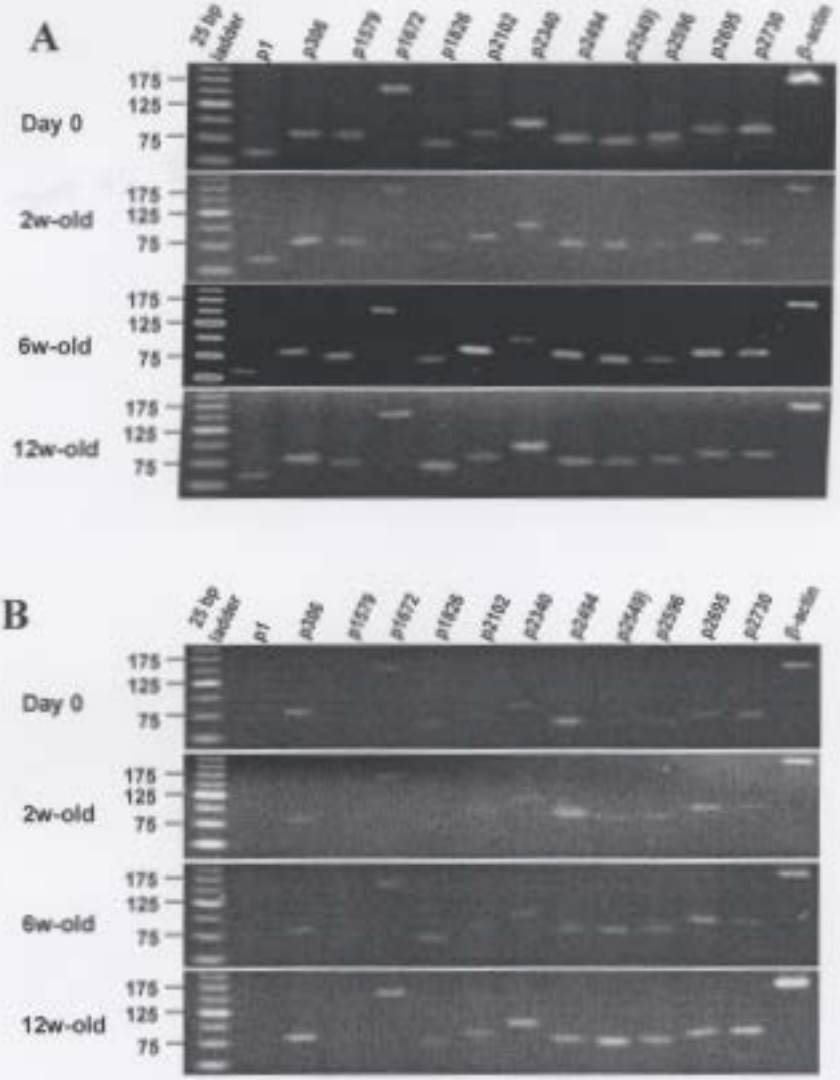

Figure 7.2. mRNA expression profile of Tg peptides in the thyroid (A) and thymus (B) of CBA/J mice at various ages.

mRNA were isolated from thyroid and thymus tissue of newborn, 2-week old, 6 week-old and 12-week old male CBA/J mice, and tested for the expression of Tg peptides. Data are representative of individual samples from 5 mice.

7.3.2 Relative expression of mRNA encoding Tg peptides among various tissues

Using the same set of primers, we subsequently determined the relative expression of Tg peptide mRNA in thyroid vs thymus, liver, and kidney using mRNA from β-actin as reference. As expected, the Tg mRNA transcripts were most prevalent in the thyroid gland, with their relative expression ranging from 33.6 ± 19.8% for p1 to 144.3 ± 49.1% for p306, **(Fig. 7.3)** perhaps reflecting the relative efficiency of the selected primer pairs for each region. mRNA transcripts for all tested sequences also showed a lower and variable expression in thymus, liver and kidney, except for the mRNA transcripts for p1 and p1579 which were detected only in the thyroid. These results (summarized in **Fig. 7.4**) suggested the presence of Tg isoforms in extrathyroidal tissues, including the thymus, that were truncated at the 5' end (within the 1-915 bp segment) and carried internal deletion(s) within a large fragment spanning bp 961-5013.

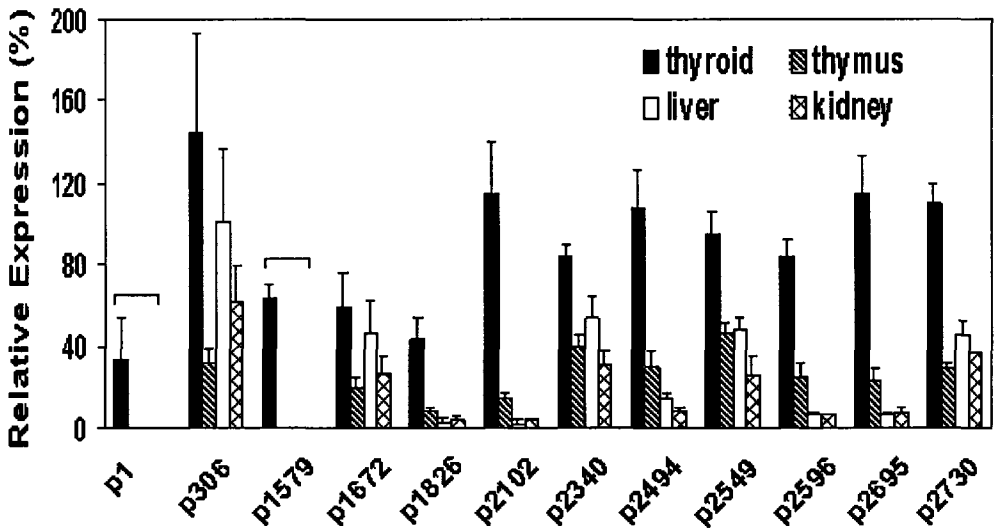

Figure 7.3. Relative expression of mRNA encoding pathogenic Tg peptides in various tissues of CBA/J mice.

Relative expression was calculated as the ratio of the relative optical density of the Tg fragment band to that of the β actin in the same sample and under similar conditions of amplification. The results are expressed as mean ± SD of values obtained from 2-3 mice.

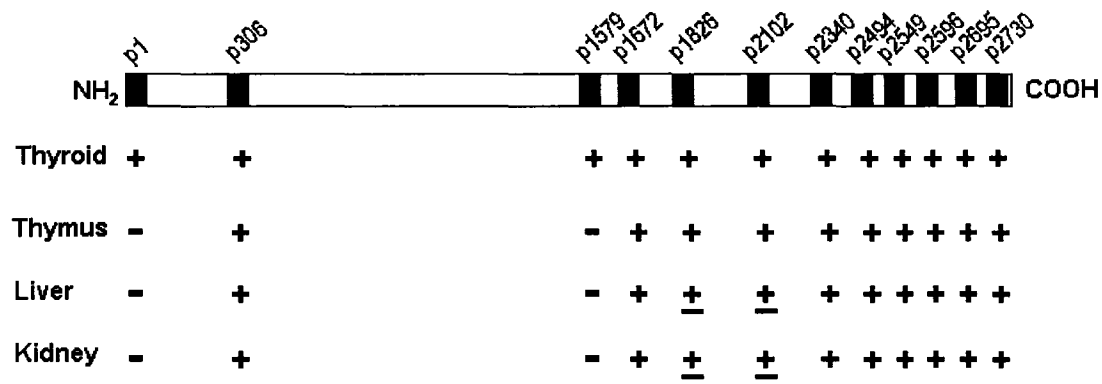

Figure 7.4. Summarized data on the relative expression of mRNA encoding pathogenic Tg peptides in various tissues of CBA/J mice.

±, relative expression varies from 0-10% among samples; - , no detection.

7.3.3 mRNA encoding a large Tg fragment (bp 961-5013) is undetectable in the mouse thymus using RT-PCR

To determine the approximate boundaries of a potential deletion between bp 961-5013 in intrathymic Tg mRNA, we used six pairs of overlapping primers to amplify, by RT-PCR, six 600-800 bp segments (A to F) spanning the 916-5133 bp region, as shown in **Fig. 7.5a**. It was found that mRNA encoding all six Tg segments was present in the thyroid but absent in the thymus of the same donor (**Fig. 7.5b**). These data strongly support the lack of a potentially large (4 kb) sequence segment within the 961-5013 bp of intrathymic Tg mRNA.

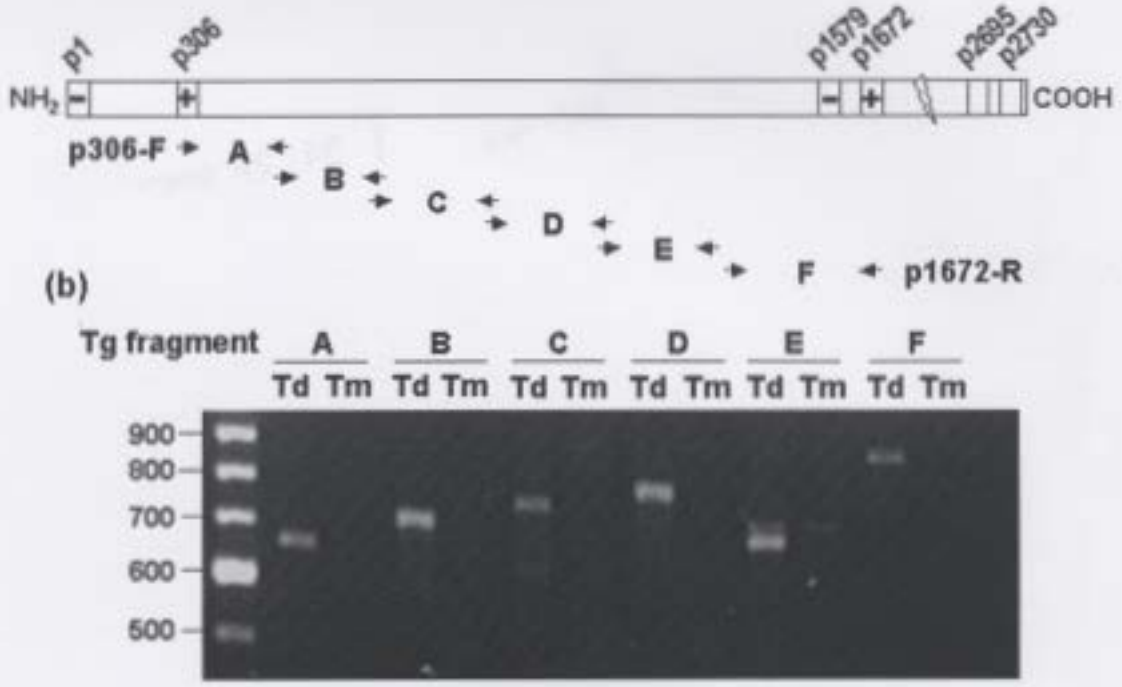

Figure 7.5. A large fragment (bp 961-5013) of Tg mRNA transcripts is undetectable in mouse thymus.

(a) A series of overlapping primers were designed and used in RT-PCR to amplify the Tg mRNA fragment encoding the a.a. sequence from p306 to p1672. (b) Visualization of PCR products in a 1.5% agarose gel stained with ethidium bromide. Td = thyroid; Tm = thymus.

7.4 DISCUSSION

In this study, we have tested the intrathymic expression of mRNA encoding 13 pathogenic peptides scattered throughout the length of the large Tg autoantigen. It was found that mRNA encoding the N-terminal peptide (aa.1-12), and the peptide (aa 1579-1591) toward the middle of the molecule, was detectable in the thyroid but not the thymus. In contrast, mRNA encoding all other peptides was easily detectable in both organs. Overlapping primer pairs, spanning the 916-5133 bp region, were subsequently found to similarly amplify RT-PCR products of the expected size in thyroid but not thymic tissue. These results suggest that Tg mRNA in the mouse thymus exists as different isoform(s), either in the form of deletion or differently sliced alternatives. Further analysis of the size of Tg transcripts in the thymus vs thyroid by northern blotting may help to differentiate these probabilities.

Our findings caution against using primers from random Tg sites to study extrathyroidal Tg gene expression by RT-PCR. Also, they indicate that apparently contrasting observations of other investigators on this issue may be explained on the basis of the Tg mRNA segment chosen for amplification. Heath et al. have reported intrathymic Tg gene expression in male PVG rats using primers amplifying the 7597-8241 bp fragment (Mor et al. 1998), whereas Mor et al. have not been able to detect Tg mRNA in thymocytes of Lewis rats (Spitzweg et al. 1999a) with primers amplifying the 532-832 bp segment, close to the N-terminus of the molecule. Furthermore, differential Tg transcripts between species may preclude RT-PCR-based extrapolations from mice to humans in regard to

intrathymic Tg expression. For example, Spitzweg et al. (Carayanniotis 2003) detected Tg gene expression in human thymus using primers amplifying the 4464-5146 bp region whereas we have been unable to detect Tg in mouse thymus via amplification of the 4735-4773 bp segment (encoding p1579) which is localized within the above region.

The presence of intrathymic Tg isoforms may have implications for mechanisms of central tolerance to Tg, since negative selection may not occur against dominant T-cell epitopes if they map within regions not represented in the thymus. Also, the hierarchy of immunodominance resulting from the processing of intrathymic Tg may be different from that of the peripheral Tg. So far, all known pathogenic T cell-epitopes in Tg have been classified as non dominant (Dai et al. 1999) and some of them are known to be produced in vitro under certain conditions e.g. after processing of Tg-Ab immune complexes (Dai et al. 2002) or processing of highly iodinated Tg (Derbinski et al. 2001, Heath et al. 1998). It is not known, however, to what extent non dominant T-cell epitopes can be generated in the thymus from endogenous Tg produced in thymic epithelial cells (Spencer 2000), or blood-borne Tg molecules that leak from the thyroid in small amounts (Klein et al. 2000). One, therefore, cannot draw correlates between the presence of intrathymic mRNA encoding a non-dominant Tg epitope and the immunogenicity of this epitope, as has been done with dominant peptides in other systems (Wan et al. 1997). For example, the Tg peptide (aa 1-12) whose mRNA is undetectable in the thymus has been found to be weakly immunopathogenic (Sellitti et al. 2000).

Our results confirm those of earlier studies reporting Tg gene expression in mouse - as well as human – kidney (Bugalho et al. 2001). On the other hand, it is not clear to what extent the detection of Tg mRNA transcripts in the liver is due to the presence of contaminant leukocytes since it has been reported that Tg is expressed in blood cells (Sellitti et al. 2000). Nevertheless, it is intriguing that the extrathyroidal expression patterns of various Tg transcripts in our study is similar among thymus, liver and kidney, i.e. they indicate lack of the 1-915 bp and 961-5013 bp segments, spanning exons 1-7, and 9-22, respectively. This concordant pattern of amplicon expression and the number of amplicons examined also supports the view that they originate from Tg mRNA, although this has not been formally shown by sequencing data. The possibility of an in situ production of Tg protein in the kidney has been previously raised (Alvino et al. 1995, Kohn 1995), but the functional properties, if any, of extrathyroidal Tg remain unknown. Tg has been reported to possess an intrinsic cAMP-dependent protein kinase activity, and it may autophosphorylate serine residues in vitro (Taurog 2000). The catalytic or ligand-binding activity of Tg probably lies with two motifs comprised of a.a. residues (154 -160) and (468-475) i.e. sites which, as our data indicate, may be missing in extrathyroidal Tg.

CHAPTER 8

FUTURE DIRECTIONS

Normal human Tg varies widely in iodine content from values as low as 0.1% to as high as 1.1%, depending on the amount of iodine intake (Rose et al. 1997, Rose et al. 2002, Ruwhof and Drexhage 2001b). Excessive iodine intake has been reported to be associated with increased prevalence of thyroid diseases in humans. Enhanced iodination of Tg conferred higher immunogenicity, probably by facilitating the generation of either iodotyrosyl-containing neoantigenic determinants (Chapter 3) or non-iodinated cryptic epitopes (Dai et al. 2002). Although enhanced iodination of Tg caused a net increase in its overall immunogenicity and pathogenicity in experimental animals (Sundick et al. 1987, Dai et al. 2002), at individual peptide level, iodotyrosyl formation may have increasing, decreasing and neutral effects on its immunogenic profile (Chapter 4). None of these newly identified and previously reported Tg peptides (Carayanniotis 2003) appeared to be immunodominant, as determined by LNC proliferative assays. Interestingly, all these peptides are expected to be present in the thyroid, as adoptive transfer of T cells specific for these Tg determinants can cause the development of EAT. These findings prompted us to hypothesize that pre-cleavage of Tg by protease or other enzymes specific in the thyroid may be required to generate those so called "cryptic" determinants. However, the current findings also brought our attention to how self tolerance against Tg – the largest A-Ag – is maintained under physiological conditions and broken upon immunological

challenge. We have reported in Chapter 7 that Tg mRNA is present in different isoform(s) in mouse thymus versus thyroid, but circulating Tg containing low iodine content can enter thymus via blood. The process via which Tg-specific autoreactive T cells are deleted, ignored or controlled by Treg is unknown. Yet, the fact that only Tg containing certain levels of iodine, but not LI-Tg, is immunopathogenic (Section 1.3.2) implies that self tolerance may be established against LI-Tg coming from circulating blood.

Another interesting finding in my studies is that SJL – but not CBA/J - mice fed on drinking water supplemented with 0.05% NaI did not promote the generation of highly iodinated Tg in both SJL and CBA/J mice, but elicited the development of goitrous hypothyroidism in SJL but not CBA/J mice (Chapter 5). The iodide-induced hypothyroidism in SJL mice was accompanied by focal infiltration of mononuclear cells in thyroid glands, but no Tg-specific autoimmune responses were observed in these hypothyroid mice. These results highlight a critical role of genetic factors in controlling the susceptibility to iodide-induced hypothyroidism, and raise our concerns about the role of anti-thyroid responses in the development of hypothyroidism. To exclude the effects of MHC genes on iodide-induced hypothyroidism, other H-2^s mouse strains, such as B10.S, will be treated with NaI-supplemented water to determine the development of hypothyroidism. In our future studies, we will try to identify the polymorphic genes which control the susceptibility to iodide-induced hypothyroidism in SJL mice. We also want to examine the mechanisms underlying the iodide-induced hypothyroidism in SJL mice.

Hypothesis 8.1: NIS polymorphisms may account for the differential effects of iodine excess on the induction of hypothyroidism.

The significant differences in development of iodide-induced hypothyroidism in female SJL vs CBA/J mice enable us to define the susceptible genetic factors underlying such phenomenon. In the current study, I have shown via RT-PCR that NIS gene is probably differentially regulated by excessive iodide in SJL vs CBA/J mice (Chapter 5). Mutations in the NIS gene in patients with congenital hypothyroidism have been previously reported to cause a defect in iodide transportation (Fujiwara 1997, Pohlenz et al. 1997). Therefore, I hypothesize that mutations or polymorphisms of NIS gene in SJL mice may lead to the induction of hypothyroidism following iodide administration. First, we need to confirm the effect of iodine on NIS expression in SJL and CBA/J mice at both mRNA and protein level, using real time-PCR and western blotting, respectively. Next, thyroid genomic DNA encoding NIS gene with the promoter region will be isolated, amplified and sequenced to define mutations or polymorphisms. If differences in encoding sequence are found between SJL vs CBA/J mice, we will test differences in the functionality of NIS alleles using COS-7 cells transfected with plasmid DNA encoding these alleles, following an established protocol (Carrasco et al. 2000).

The expected result from this experiment is that COS-7 cells transfected with plasmid DNA encoding NIS of SJL mice will take up significantly more $Na^{125}I$ than those transfected with plasmid DNA encoding NIS of CBA/J mice. These results may support

the view that intrathyroidal iodine in SJL mice will reach and will be maintained at high levels after iodide administration, preventing escape from "the Wolff-Chaikoff effect". The high concentration of intrathyroidal iodide may also exert direct toxic effects on thyrocytes, causing cell necrosis. As suggested in Chapter 6, necrotic thyrocytes in situ may facilitate the induction of EAT by triggering DC maturation (*Hypothesis 8.2*). Alternatively, the experimental results may not agree with the working hypothesis. Polymorphisms in other molecules in the process of iodine organification may account for the susceptibility to iodide-induced hypothyroidism.

Hypothesis 8.2: Iodide-induced thyrocyte necrosis in situ releases both autoantigen and maturation signals to intrathyroidal DC, which activate autoreactive T cells leading to EAT.

In the thyroid, iodide is concentrated by a factor of 20-to 40-fold with respect to its concentration in the plasma under physiologic conditions (Li et al. 1993). Elevated intrathyroidal iodine, following enhanced iodine intake, can recruit more DC into the site, as observed in BB/W rats (Mooij et al. 1994b). Moreover, it was reported that thyroid hormones T3 or T4, or HI-Tg, increased the transition of human peripheral blood monocytes into veiled/dendritic cells ex vivo (Follis 1959, Li and Boyages 1994, Many et al. 1995, Bagchi et al. 1995). In several animal studies, it has been postulated that a direct toxic effect of iodine on thyrocytes in situ is involved in triggering thyroid-specific autoimmune reactions (Many et al. 1992). In the current study, we have shown that DC

exposed to necrotic thyrocytes readily present Tg released from the necrotic thyrocytes and activate thyroiditogenic T cells. Collectively, these data raise the hypothesis that iodine -induced thyrocyte necrosis could serve as an initial event that triggers DC maturation and antigen presentation, thus leading to AITD.

Cell necrosis occurred only in < 10% of the cultured thyrocytes in the presence of 10^{-3} M NaI (Fradkin and Wolff 1983), which is 10,000 times higher than normal plasma iodine levels in healthy population (Williamson et al. 2002). The low necrotic rate hampers the ex vivo generation of large numbers of thyrocytes necrotized by iodide. To circumvent this problem, we will investigate the effects of thyrocytes necrotized by iodide on thyroid-resident DC in vivo. Thyroid-resident DC are expected to take up A-Ags, such as Tg, released from necrotic thyrocytes, while they receive maturation signals from the necrotic cells. Following migration to the thyroid-draining lymph nodes, DC will present the thyroid autoantigens to T cells, thus eliciting thyroid autoimmune disease.

To test this hypothesis, various doses of NaI (0.1%, 0.5%, 2.5%) will be administered to SJL and CBA/J mice for 10 weeks, and $CD11c^+$ DC will be purified from a single cell suspension of thyroid-draining lymph nodes using MACS separation columns (Miltenyi Biotec, Auburn, CA). To enrich DC population, these animals will be injected i.p. daily for 10 days before sacrifice with 10 μg Flt3 ligand, which expands all DC populations without inducing their activation (Voorby et al. 1990, Many et al. 1995, Hala et al. 1996). DC maturation markers (MHC, CD80, CD86, CD40) will be assessed by flow cytometry.

Mice maintained with normal water will be used as controls. Upregulation of surface markers such as MHC, CD80, CD86 and CD40 on DC from iodide-treated mice versus those from controls will indicate the phenotypic maturation of the DC. If DC in the thyroid-draining LNs from experimental mice express higher levels of MHC and costimulatory molecules than those from control mice, we will continue to examine the capability of such DC in activating thyroid-specific autoreactive T cells in naïve recipient mice upon i.v. transfer.

Hypothesis 8.3: Thyroid-infiltrating DC or macrophages secrete cytokines, such as IL-1β, which suppress the release of thyroid hormones from thyrocytes.

In most animal models of SAT, thyroid infiltration starts with the local accumulation of DC and macrophages (Gu et al. 1995). In 1994, it was reported that reduction of pancreas-infiltrating DC and macrophages via anti-CR3 mAb (5C6) treatment resulted in a consistent (25%) increase in the growth of islet cells, as well as a decrease in blood glucose level, an indicator for enhanced insulin secretion (Gaytan et al. 1995, Hayashi et al. 1994). Similarly, adult rats - depleted of testicular macrophages using liposome-entrapped dichloromethylene diphosphonate – showed a significant increase in serum level of testosterone (Simons et al. 1998). In vitro, splenic DC were found to inhibit the growth of freshly isolated rat thyrocyte follicles in an IL-1β-dependent manner (Gu et al. 1995). Based on these observations, it has been postulated that DC and macrophages

infiltrating the endocrine organs may exert a downregulatory effect on the growth and function of endocrine cells.

In our model of iodide-induced hypothyroidism in SJL mice (Chapter 5), we observed the coexistence of thyroid infiltration and hypothyroidism. However, the phenotype and function of the thyroid-infiltrating cells remains unknown. First, immunohistochemical staining of thyroid cryosections using anti-CD11b and anti-CD11c Abs will be performed to determine the presence of DC and macrophages in the thyroid lesions following iodine administration. Second, if DC and macrophages are found, we will examine whether DC and macrophages contribute to the decrease in the serum thyroid hormone levels. We will deplete these cells by modified Gu's regimen (Jung et al. 2002). Briefly, SJL mice will be depleted of DC and macrophages by i.p. injection of anti-CD11b 5C6 mAbs (500 μg/mouse, 3 x per week for 1 month). The mice will then be placed on water supplemented with 0.05% NaI, while they continue receiving 5C6 mAbs for another 10 weeks. Normal SJL mice treated similarly with or without NaI will be used as controls. At the end of the period, thyroid histology and serum thyroid hormones will be examined. The expected results will be that SJL mice depleted of DC and macrophages develop less or no hypothyroidism and thyroidal infiltration. If this hypothesis is supported by the experimental results, the role of iodine in this model will be mostly likely through its toxic effects on thyrocytes, and signals released from necrotic thyrocyte will recruit DC and macrophages, leading to hypothyroidism.

The above protocol depletes both DC and macrophages, to specifically examine the role of DC in iodide-induced hypothyroidism, a transgenic mouse line carrying a transgene encoding diphtheria toxin receptor (DTR) under control of the murine CD11c promoter will be used (Naglich et al. 1992). These mice were generated based on the strategy that murine DC will undergo apoptosis upon endocytosis of DT via its receptor (Jung et al. 2002). To use these transgenic mice in our model, we need to backcross the DTR-CD11c transgenic mice, generated in Balb/c or C57bl/6 background, to SJL mice. To determine whether DC are required in early stages for the initiation of iodide-induced hypothyroidism, DTR-CD11c transgenic SJL mice will be depleted of DC via i.p. injection of 100 ng of DT at d0 (Dunn and Dunn 1988, Dunn et al. 1991), and placed on water supplemented with 0.05% NaI for 10 weeks while continuously receiving DT for every 3 days. Other groups of mice will receive DT injection 2 or 4 weeks, respectively, after iodide administration. Mice treated similarly with NaI but not receiving DT will be used as controls. These mice will then be sacrificed to examine thyroid histology and thyroid hormone levels in the sera. If DC is required for early events during iodide administration, only mice receiving DT before or shortly after iodide administration will develop less hypothyroidism. As mentioned above, the toxic effects of iodine on thyrocytes will be considered as an initiation event to recruit DC and macrophages, leading to hypothyroidism.

Hypothesis 8.4: Precleavage of Tg by intrathyroidal proteases is required for generation of Tg cryptic determinants

In the human thyroid, cysteine protease - cathepsins B and L, as well as aspartic protease - cathepsin D, are the major lysosomal endopeptidases participating in Tg degradation (Dunn et al. 1991). The cleavage of Tg by these enzymes creates Tg fragments which may facilitate the generation of cryptic determinants. To test this hypothesis, these proteases will be incubated at their pH optimum at 37°C with Tg at different ratios of enzyme/substrate according to an established protocol (Ishisaka et al. 1998). Digestion products will then be added to the coculture of bone marrow-derived DC and T cell hybridoma clones specific for Tg cryptic determinants. Undigested Tg will be used as negative controls and free peptides will be used as positive controls. The activation of T cell hybridoma will be assessed by IL-2 secretion using CTLL-2 cell proliferation. To confirm the role of candidate proteases in the generation of Tg cryptic determinants, specific inhibitors for cathepsin B (CA-074 Me) and L (Z-FY(t-Bu)-DMK) will be added in the culture at optimal doses according an established protocol (Casiano et al. 1998, Rodenburg et al. 2000).

Hypothesis 8.5: Precleavage of Tg by cell death-activated proteases facilitates the generation of Tg cryptic determinants

It has been proposed that proteolysis of A-Ags by activated caspases (cysteine aspartases) during apoptosis, or distinct but unknown proteases during necrosis, leads to the

generation of neoantigenic determinants (Sauter et al. 2000). With the availability of primary thyrocytes, we are able to test the role of cell death on the processing and presentation of cryptic Tg determinants. Following Sauter's protocol (Ip and Lau 2004), cell apoptosis will be induced by UV irradiation and necrosis will be induced by repeated freezing and thawing (as seen Chapter 6). Cell death will be evaluated using an apoptosis detection kit (Immunotech, Marseilles, France). Briefly, cells will be stained with FITC-labeled annexin V and propidium iodide (PI) according to the manufacturer's protocol, and dead cells will be detected by flow cytometry. Apoptotic cells at early stage will be annexin V^+ PI^-, and necrotic cells will become annexin V^+ PI^+ (Ishisaka et al. 1998). In addition, necrotic but not apoptotic cells incorporate trypan blue in the cytoplasm due to the loss of their membrane integrity (Current Protocol in Immunology, section 3.17).

BM-DC will then be exposed to apoptotic or necrotic thyrocytes, respectively, at 2:1, 5:1 or 10:1 ratios for overnight. To determine the efficiency of phogocytosis by DC, thyrocytes will be labeled with the red fluorescent dye PKH26 before death and immature BM-DC will be labeled with green fluorescent dye PKH67, according to the manufacturer's protocol (Sigma-Aldrich). Following coculture, phagocytosis of dead cells by DC will be defined by the percentage of DC with double-positive fluorescent signals using flow cytometry. The generation and presentation of Tg cryptic determinants on the surface of DC will be assessed by T cell activation assay using T cell hybridoma clones specific for Tg cryptic determinants. To confirm the function of caspases and to identify the candidate protease responsible for the generation of Tg cryptic determinants, protease

inhibitors, such as leupeptin, pepstatin A or aprotinin, will be added into the DC : dead thyrocytes coculture at optimal doses according to an established protocol. These experiments will support the fact that cryptic Tg determinants are normally present in thyroid glands, and provide evidence demonstrating that cryptic determinants can be generated when processing of Tg is altered under certain conditions.

In summary, the future work is focused to explore the mechanisms underlying iodide-induced hypothyroidism and EAT by determining the genetic susceptibility (hypothesis 8.1), and effects of local inflammation caused by iodide (hypothesis 8.2 & 8.3). These experiments will provide information on how genetic and environmental factors may interplay with each other to cause EAT and/or hypothyrodism in mice, and help us in understanding the etiology of HT in humans and its prevention. We will also continue to define parameters that influence the processing of Tg in thyroid and conditions under which cryptic Tg determinants may be generated (hypothesis 8.4 & 8.5). These findings may provide an explanation to our current experimental results that autoreactive T cells specific for a cryptic Tg peptide can home to the thyroid gland and cause EAT upon adoptive transfer. Meanwhile, they may also have implications on how self tolerance to Tg is broken down during disease.

GLOSSARY

Term	Definition
ADCC	= Antibody-dependent cell-mediated cytotoxicity. The lysis of Ab-coated target cells when interacting with Fc receptor on the surface of effector cells.
Adjuvant	A substance that, when mixed with antigen, increases its immunogenicity by provoking local inflammation.
Alleles	One of a number of slightly different DNA sequences that code for the same gene.
Anergy	A state of lymphocyte non-responsiveness to specific antigen.
Antibody	A Y-shaped glycoprotein produced by B lineage plasma cells. Each antibody recognizes a specific antigen that is either soluble or fixed in a tissue or on a cell surface.
Antigen	A substance that can bind to the antigen receptor of a B cell. This binding, however, does not necessarily lead to B cell activation.
Antigen presenting cells (APC)	Cells expressing MHC class II molecules on their surface thus capable of presenting peptide to $CD4^+$ T cells. Professional APCs such as DC, B cells and macrophages, express high levels og MHC class II.

Term	*Definition*
Apoptosis	Also called "programmed cell death", is a normal process of deliberate life relinquishment by a cell in a multicellular organism. It is mediated by certain intracellular proteases that cause the orderly breakdown of cell nucleus and DNA, without release of internal contents.
Autoantibody	Antibodies produced by plasma cell and directed against one or more of the host's own proteins.
Autoantigen	A self antigen that can be recognized by either autoantibody or self-reactive T cells.
Autoimmune disease	A pathophysiological state of an organism due to the failure of its immune system to recognize its own constituent parts as "self". It involves either a specific organ (organ-specific autoimmune disease) or many tissues of the body (systemic autoimmune disease).
Congenic	Two individual animals that carry identical alleles at all but one locus.
Costimulation	The second signal required for optimal lymphocyte activation and differentiation. Supplied by engagement of CD28 by B7-1/B7-2 (T cell) and CD40 by CD40L (B cell).
Costimulatory molecules	Molecules involved in mediating the costimulation of a cell, such as CD28 and B7-1/B7-2, or CD40 and CD40L.

Term	*Definition*
Cross-presentation	Presentaion of peptides from exogenous antigens on the MHC class I molecules of DC and macrophage.
Cross-priming	Activation of CD8 T cells through cross-presentation of exogenous antigens by DC or macrophage.
Epitope	A small region of a macromolecule that is specifically recognized by the antigen receptor of a B or T lymphocyte.
Experimental autoimmune thyroiditis (EAT)	Animal model of Hashimoto's thyroiditis in humans, is a T cell-mediated autoimmune disease, characterized by destruction of thyroid follicles by infiltrating inflammatory cells. Induced by immunization with thyroglobulin (Tg) or Tg peptides in complete Freund's adjuvant.
Hashimoto's thyroiditis (HT)	= Autoimmune thyroiditis (AT) or lymphocytic thyroiditis (LT). A type of autoimmune disease in which the immune system attacks and destroys the thyroid gland, leading to the development of hypothyroidism (elevated TSH and low T4).
Homeostasis	The tendency of a biological system to maintain a steady state.
Hybridoma	Immortalized cells that have been engineered to produce a desired antibody or express a specific T cell receptor; created by fusion of a myeloma or lymphoma cell with an activated B or T cell, respectively.

Term	*Definition*
Hypothyroidism	A pathophysiological state caused by insufficient production and/or release of thyroid hormone by the thyroid gland (low serum T4 level). The most common cause is Hashimoto's thyroiditis.
Immunogenicity	The ability of a particular substance to provoke an immune response.
Inbred mice	Strains of mice in which all members are genetically identical at virtually all loci and each mouse is homozygous at all loci.
Iodination	The substitution or addition of iodine atoms in organic compounds.
Monoclonal antibody	Antibodies that are identical with a single, known specificity. Produced by a single B cell clone.
Necrosis	An accidental cell death due to injury, infection or inflammation. The cell loses its integrity and releases intracellular components.
Priming	The first encounter of a naïve lymphocyte with specific antigen.
Self tolerance	Lack of an immune response to self antigens in an individual's tissues.
Syngeneic	Animals that have the same alleles at all genetic loci. In some cases, "syngeneic" is used to refer to genetic identity at the MHC loci only.

Term	*Definition*
Th cells	Helper T cells that recognize peptides presented in the context of MHC class II molecules on APCs. They may differentiate into either Th1 or Th2 in phenotype. Th1 cells secrete IFNγ and IL-2, and induce production of IgG1 and IgG3 in humans. Th2 cells secrete IL-4, IL-5 and IL-10, and induce production of IgA, IgE and IgG4 in humans.
Transgenic (Tg) mouse	A mutant mouse created by the introduction of a desired gene (the "transgene") into the genome of a whole mouse embryo. The transgene is expressed along with the recipient's own genes.
Wolff-Chaikoff effect	Administration of large amounts of iodide into certain species results in a temporary decrease in the formation and release of thyroid hormone, due to inhibited iodide organification.

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