Studies on the immunoregulation of autoimmune thyroiditis

by

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ABSTRACT

Autoimmune thyroiditis is a chronic disease characterized by mononuclear cell infiltration in the thyroid gland and production of anti-thyroglobulin (Tg) antibodies. In this thesis, we have used the murine models of experimental autoimmune thyroiditis (EAT) and iodide-accelerated spontaneous autoimmune thyroiditis (ISAT) to investigate the immunoregulation of the disease.

In the EAT model, we have examined whether putative $H2A^k$-binding Tg epitopes, located adjacent to cathepsin cleavage sites within mouse Tg would have immunopathogenic properties. The peptides, p2369 and p2439, elicited significant proliferative T cell responses and production of IL-2 and IFN-γ in recall assays using lymph node cells from peptide-primed mice. Both peptides were pathogenic in CBA/J mice after direct challenge with the peptide in CFA and by adoptive transfer of peptide-primed lymph node cells into naïve recipient hosts.

In the ISAT model, we have examined whether NOD.$H2^{h4}$ thyrocytes are highly prone to iodide-mediated apoptosis since such an event could theoretically trigger ISAT in this strain. Our data showed that NOD.$H2^{h4}$ thyrocytes cultured with low NaI concentrations had significantly higher apoptotic rates compared to CBA/J thyrocytes. Increased apoptosis was associated with an impaired control of oxidative stress mechanisms since the expression of only two genes involved in ROS metabolism and/or anti-oxidant

function were upregulated in iodide-treated NOD.H2^{h4} thyrocytes vs. 22 genes in iodide-treated CBA/J thyrocytes. Next, we examined the immunopathogenicity of the Tg epitope, a.a. 2549-2560 (T4p2553) in NOD.H2^{h4} mice. The epitope was immunopathogenic in this strain after direct challenge with peptide in CFA. In ISAT, peptide-specific proliferative responses were recorded using only cervical lymph node cells (CLNC) from iodide-fed mice before the onset of the disease. Also, selective enrichment of CD4^{+} IFN-γ^{+} T4p2553-specific cells was observed among CLNC and intrathyroidal lymphocytes. T4p2553 was equally detectable on dendritic cells from CLNC regardless of iodide intake whereas spontaneous T4p2553-specific IgG responses were not detectable. Our data identified for the first time a Tg T-cell epitope as a spontaneous target in ISAT.

Lastly, we have examined whether a high salt diet can exacerbate autoimmune thyroiditis because previous studies by other groups had suggested this diet as a predisposing factor for autoimmune diseases in general. Our data showed that high salt intake failed to significantly affect the incidence and severity of SAT in NOD.H2^{h4} mice or EAT in Tg-primed C57BL/6J and T4p2553-primed CBA/J mice relative to mice on a normal diet. No difference was detected at the Tg-specific IgG levels between the two groups. These data demonstrate that a high salt diet is not a risk factor for all autoimmune diseases.

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a.a.	Amino acid
Ab	Antibody
AITD	Autoimmune thyroid disease
APC	Antigen presenting cell
BM	Bone marrow
CD	Control diet
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CLN	Cervival lymph nodes
CLNC	Cervical lymph node cells
CS	Cornell Strain
DC	Dendritic cells
DIT	Diidotyrosine
DMEM	Dulbecco's modified Eagle's medium
EAE	Experimental autoimmune encephalomyelitis
EAT	Experimental autoimmune thyroiditis
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

GM-CSF	Granulocyte macrophage colony stimulating factor
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen
HSD	High salt diet
HT	Hashimoto's thyroiditis
hTg	Human thyroglobulin
hTPO	Human thyroid peroxidase
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon-γ
I.I.	Infiltration index
IL	Intrathyroidal lymphocytes
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-17	Interleukin-17
IL-23R	Interleukin-23 receptor
i.p.	Intraperitoneal
ISAT	Iodide-accelerated spontaneous autoimmune thyroiditis
I-Tg	Highly iodinated thyroglobulin

i.v.	Intravenous
kDa	Kilodalton
LN	Lymph nodes
LNC	Lymph node cells
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MIT	Monoiodotyrosine
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
mTg	Mouse Tg
mTPO	Mouse thyroid peroxidase
NIS	Sodium iodide symporter
NK	Natural killer
NOD	Non-obese diabetic
NP	Nucleoprotein
NPL	Nitrophenyl
Nrf2	Nuclear factor erythroid 2
OS	Obese strain
OVA	Ovalbumin
PBS	Phosphate buffered saline
pTg	Porcine thyroglobulin

RBC	Red blood cells
ROS	Reactive oxygen species
SAT	Spontaneous autoimmune thyroiditis
s.c.	Subcutaneous
S.I.	Stimulation index
T0	Thyronine
T3	Triidothyronine
T4	Thyroxine
Tg	Thyroglobulin
TNF-α	Tumor necrosis factor-α
TPO	Thyroid peroxidase
Treg	Regulatory T cells
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone-receptor
TCR	T cell receptor
WT	Wild type

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Chapter 1

Introduction & Overview

1.1. Thyroid gland and function

The thyroid is the largest endocrine gland in humans and it is located in the middle of the neck. It consists of two lobes that are connected by the isthmus, normally located on the second or third ring of the trachea. The thyroid is comprised of follicles and a small number of parafollicular calcitonin-producing cells, the C-cells, which make up about 10% of the thyroid mass. The thyroid follicles are composed of an outer layer of epithelial cells called thyrocytes, while the interior of the follicle, the lumen, contains thyroglobulin (Tg), a protein produced only by thyrocytes. Tg acts as a prohormone for the thyroid hormones thyroxine (T4) and triidothyronine (T3). T4 and T3 are eventually secreted by thyrocytes into the bloodstream, following Tg proteolysis in the thyrocyte (Santisteban 2005).

The thyroid hormones are involved in the regulation of the cellular metabolic rate, heart, digestive and muscle function, brain development and bone maintenance (Chi et al. 2013). Due to their crucial role in many biological activities, the levels of the thyroid hormones are regulated by a negative feedback loop (Santisteban 2005). Disturbance of this regulatory mechanism can lead to overproduction or decreased production of the thyroid hormones, causing the pathological conditions hyperthyroidism and hypothyroidism, respectively.

1.2. Hashimoto's thyroiditis

One of the most common causes of hypothyroidism is the autoimmune disease of the thyroid gland called Hashimoto's thyroiditis (HT) (Farwell, Braverman 1996), also known as lymphocytic thyroiditis or struma lymphomatosa. HT, described for the first time in 1912 by Dr. Hakaru Hashimoto (Hashimoto 1912), is a chronic disease characterized by thyroid infiltration of hematopoietic mononuclear cells, mainly lymphocytes, plasma cells and macrophages, as well as by the presence of serum antibodies (Abs) against Tg and thyroid peroxidase (TPO) (Caturegli et al. 2014). Intrathyroidally, lymphocytes can either form lymphoid follicles resembling germinal centers or come in close contact with thyrocytes to mediate their destruction (Caturegli et al. 2014). Tissue injury can be mediated by cytotoxic T cells or by cytokines release by T cells (Weetman, McGregor 1994). Also, thyroid-specific Abs can cause injury either through complement activation (Chiovato et al. 1993) or by ab-dependent cellular cytotoxicity (Bogner et al. 1984). Patients with HT can display a wide spectrum of clinical symptoms ranging from formation of germinal centers in the thyroid, fibrosis, thyroid cell hyperplasia and/or enlargement of the thyroid gland (goiter), usually leading to hypothyroidism (Weetman 1996).

HT can occur alone or in association with other autoimmune diseases like type I diabetes mellitus or Sjögren's syndrome. Also, HT has been associated with papillary thyroid cancer in 0.5 to 30% of the cases (Caturegli et al. 2014). The prevalence of HT is

reported as 8 cases per 1000 persons (Jacobson et al. 1997) and it is 8 times more frequent in women than men in white and Asian populations (Caturegli et al. 2014).

So far, there is no cure for HT. Patients with hypothyroidism receive synthetic T4 orally, the only effective drug available, daily for life. In cases where severe cervical compression has occurred or thyroid cancer is also present with HT, thyroidectomy is performed (Caturegli et al. 2014). Therefore, treatment for HT deals only with the symptoms of the disease and not with its pathogenesis.

1.3. Autoantigens in autoimmune thyroiditis

1.3.1 Thyroglobulin

Tg is a large dimeric glycoprotein with a molecular mass of 660 kiloDalton (kDa) and comprises the most abundant protein of the thyroid gland (~75% of the protein content) (Vali et al. 2000). Tg is produced in the cytoplasm of thyroid epithelial cells and is stored inside the follicular lumen at concentrations as high as 500 mg/ml (Berndorfer et al. 1996, Herzog et al. 1992). Two known physiological functions of Tg are: 1) to serve as a scaffold protein for the synthesis of the thyroid hormones, T4 and T3; and 2) to store available iodide.

Tg consists of two identical polypeptide chains each of which has 2749 amino acids (a.a.) (Carayanniotis 2003). This protein is highly conserved among different species; the identity between human and bovine is 77.3%, human and mouse 71.8% and 73.5%

between bovine and mouse (Vali et al. 2000). The N-terminal region of Tg (residues 1-2170) contains three types of cysteine-rich repetitive domains, type I, II and III with unknown functions (Dunn J.T., Dunn A.D. 2000). The type I domain, about 60 a.a. long, is repeated 10 times and may participate in Tg degradation by reversible inhibition of proteases (Molina et al. 1996). The type II domain, 14-17 a.a. long, is repeated 3 times, whereas the type III domain is repeated 5 times. The C-terminal 250 a.a. segment of the protein has a high content of tyrosine residues compared to the other regions. This part of the protein shows a striking similarity to the enzyme acetylcholinesterase, a member of the type B carboxylesterase family (Dunn J.T., Dunn A.D. 2000). Interestingly, the lack of homology between the N- and C-terminal regions as well as the difference in the size of the introns between the two regions suggest that they may have been derived from different genes that were either fused or condensed to generate the Tg gene (Mori et al. 1987).

The Tg gene in humans, mice, rats and cows is located on chromosomes 8, 15, 7 and 14, respectively (Dunn J.T., Dunn A.D. 2000). In humans, the Tg gene spans 300 kb and contains 48 exons, which are separated by introns of various sizes, up to 64 kb (Mendive et al. 2001). Expression of the Tg gene is highly restricted to thyroid cells and is controlled by the thyroid-specific transcription factors 1 and 2 as well as the paired-box protein, via binding to the highly conserved promoter region (Rubio, Medeiros-Neto 2009). Exogenous factors such as thyrotropin (TSH), insulin and insulin-like growth factor-1 have been shown to stimulate Tg gene expression whereas factors such as epidermal growth factor, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and

retinoic acid inhibit Tg gene expression (Dunn J.T., Dunn A.D. 2000). Interestingly, Tg itself has been shown to limit expression of Tg as well as TPO and the sodium iodide symporter (NIS) gene in FRTL-5 cells (Suzuki et al. 1998).

The biosynthesis of Tg occurs within the cytoplasm of thyrocytes. The full length of the Tg messenger RNA (mRNA) transcript is 8.7 kb (van de Graaf et al. 2001) and is translated on polyribosomes bound on the endoplasmic reticulum (ER) of thyrocytes (Dunn J.T., Dunn A.D. 2000). The newly synthesized protein undergoes several posttranslational modifications, such as glycosylation, sulfation, phosphorylation (Vali et al. 2000), in different intracellular organelles before it acquires the proper dimeric conformation and becomes stable. First, Tg monomers are transported to the ER via their 19 a.a. signal peptide in the N-terminus. In this organelle, the first carbohydrates are added to Tg and the protein undergoes dimerization with a number of enzymes such as protein disulfide isomerase, peptydylpropyl isomerase as well as molecular chaperones facilitating the folding of the protein; misfolded protein is transferred back to the cytoplasm for degradation. After formation of stable dimers, Tg is transported to the Golgi complex for incorporation of the final carbohydrates and sulfation of these units. Phosphorylation of Tg also occurs in the cytoplasm of thyrocytes but the organelle in which this modification occurs has not been defined. At this stage, a stable form of Tg is transferred via exocytotic vehicles to the apical surface of the cells for iodination and generation of thyroid hormones (Dunn J.T., Dunn A.D. 2000).

Inside the lumen, iodination of Tg occurs in certain tyrosines (25-40 tyrosines out of 140 available) forming monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues (Vali et al. 2000). The iodination reaction is catalyzed by TPO located at the luminal surface in the presence of hydrogen peroxide. TPO catalyzes the intramolecular coupling of two DIT leading to T4 formation or one DIT and a MIT leading to T3 formation in Tg (Arvan, Di Jeso 2005). The thyroid hormone reaction generates T4 or T3 at specific sites, called acceptor or hormonogenic sites, while a dehydroalanine is formed at the iodotyrosine residue that contributed the outer ring, called donor site. It is unknown whether intramolecular coupling occurs in an intrachain or interchain fashion or both. So far, four major hormonogenic sites have been identified in the Tg monomer; sites A, B, C and D, located at the 5^{th}, $2{,}554^{th}$, $2{,}747^{th}$ and $1{,}291^{st}$ residue of Tg. In addition, three minor sites, designated as G, N and R, may be generated in certain species under variable physiological conditions, i.e. iodide availability (Dunn J.T., Dunn A.D. 2000, Arvan, Di Jeso 2005).

After the thyroid hormones have been generated within Tg, the protein is reabsorbed by the thyrocytes and undergoes extensive proteolysis to allow the release of T4 and T3 from its polypeptide backbone. Proteolysis of Tg starts within the lumen by cathepsins B, K and L allowing the solubilization of Tg and extracellular release of T4 (Friedrichs et al. 2003). Subsequently, thyrocytes take up Tg via receptors expressed on their apical surface or by macropinocytosis or by a combination of both in endocytic vehicles (Dunn J.T., Dunn A.D. 2000). These vehicles fuse with lysosomes and degradation of Tg continues. Lysosomal Tg proteolysis is a complex procedure since many pH-dependent

enzymes have been reported to participate in the process: the cysteine endopeptidases, cathepsins B, L, H, S (Dunn et al. 1991, Nakagawa, Ohtaki 1984, Petanceska, Devi 1992), the aspartic endopeptidase cathepsin D (Dunn, Dunn 1982) and the exopeptidases dipeptidyl II and lysosomal dipeptidase I (Dunn et al. 1996). Studies, however, have highlighted the role of cathepsins B, L and D in Tg processing, since specific inhibition of all these proteolytic enzymes blocked the release of iodopeptides from rabbit [^{125}] Tg incubated with lysosomal extracts from human thyroids by 80-90% (Dunn et al. 1991). Once thyroid hormones are released from Tg, they are secreted from the basal cell membrane into the bloodstream. Intracellular Tg fragments are deiodinated by an iodotyrosine-specific deiodinase (Rosenberg, Goswami 1979) and iodide is either reused for hormone synthesis or it enters the circulation.

Tg uptake by thyrocytes can follow an alternative pathway mediated by megalin, a receptor expressed on the apical surface of thyrocytes. Tg internalized by megalin is transported from the apical to the basal surface of the cell through transcytosis, avoiding lysosomal processing (Marino et al. 2001). Tg is finally released to the bloodstream reaching concentrations of 5, 10 or 133 ng/ml in humans, mice and rats respectively (Carayanniotis, Rao 1997). The presence of Tg in the circulation shows that Tg is not a sequestered antigen.

1.3.2. Thyroid peroxidase and thyrotropin receptor

TPO is a thyroid enzyme that catalyzes oxidation of iodide, its incorporation to Tg as well as the intramolecular coupling of MIT and DIT leading to T4 and T3 generation within Tg. TPO is a glycosylated homodimer (~ 107 kDa) containing a heme group and it is located at the apical membrane of thyrocytes (McLachlan, Rapoport 2007). Purification of TPO has been challenging, since this protein is not as abundant as Tg within the gland. TPO has been purified from human thyroid tissue (Czarnocka et al. 1985) as well as expressed as recombinant protein in Chinese hamster ovary cells, insect cells and yeast cells (Taurog 2000). Recently, mouse TPO was expressed in Chinese hamster ovary cells after transfection with a plasmid carrying complementary DNA (cDNA) for full length TPO (Chen et al. 2010). TPO is involved in HT and anti-TPO Abs are used as a serological marker for diagnosis of the disease since approximately 95% of patients develop them, whereas these Abs are rarely detected in healthy individuals (Caturegli et al. 2014).

TSH, a critical factor for the function and survival of thyrocytes can be sensed through the TSH receptor (TSHR) localized to the basal membrane of the epithelial cell. TSHR contains a glycosylated extracellular region, a transmembrane domain and an intracellular carboxyterminal region. The extracellular domain of the protein is connected to the transmembrane region via the so called hinge region (Rapoport 2000). Patients with Graves' disease develop TSHR-stimulating and/or inhibiting Abs that stimulate

overproduction of thyroid hormones leading to hyperthyroidism (Rapoport, McLachlan 2007). Abs against TSHR have also been reported to exist in HT patients (Weetman, McGregor 1994).

1.4. Animal models of autoimmune thyroiditis

To advance the understanding of HT pathogenesis, animal models of the disease have been developed to examine the therapeutic potential of various treatments and to facilitate the study of individual parameters contributing to the disease. In the field of thyroiditis, there are two animal models: the model of experimental autoimmune thyroiditis (EAT) in which the disease is induced in animals; and the model of spontaneous autoimmune thyroiditis (SAT) in which the disease develops spontaneously. In this thesis, both models have been used to examine mechanisms involved in the immunoregulation of the disease.

1.5. Experimental autoimmune thyroiditis

1.5.1. Methods of induction

In 1956, Rose et. al. reported for the first time that rabbits immunized with whole thyroid extract in Freund's complete adjuvant (CFA) produced thyroid-specific Abs in their sera and mononuclear cell infiltration was observed in their thyroid glands (Rose, Witebsky 1956). Using the similar approach, EAT was also induced in a variety of species; in mice (Rose et al. 1971), monkeys (Rose et al. 1966), dogs (Terplan et al. 1960), guinea pigs (Terplan et al. 1960), rats (Jones, Roitt 1961) and chickens (Jankovic, Mitrovic 1963). Most attention however has been given to mouse models because of: a) the availability of

immunological reagents; b) the well known genetics and availability of inbred mouse strains; and c) the relative ease and cost-effectiveness of mouse breeding and maintenance. In the following paragraphs, I will focus on the parameters involved in induction of EAT in mice.

EAT is induced in genetically susceptible mice either after direct immunization with thyroid autoantigens in adjuvant or after adoptive transfer of activated thyroid autoantigen-specific T cells into naïve recipients. The most commonly used protocol for direct induction of EAT uses Tg or Tg peptides as thyroid autoantigen. Mice are immunized with Tg in the presence of an adjuvant; CFA (Rose et al. 1971) and lipopolysaccharide (LPS) (Esquivel et al. 1977) are the most commonly used but muramyl dipeptide (Kong et al. 1985) and poly(A:U) (Esquivel et al. 1978) have also been shown to have adjuvant properties. EAT has also been induced in C3H/Anf mice in the absence of adjuvant by repeated immunizations with mouse Tg (mTg) (Eirehewy et al. 1981). Furthermore, immunization with mTg conjugated to anti-IA^k monoclonal Ab (mAb) in the absence of adjuvant elicited Tg-specific IgG, but no thyroid lesions in CBA/J mice (Balasa, Carayanniotis 1993).

Other autoantigens have also been used to directly induce EAT in susceptible mice. Porcine TPO in CFA induced EAT in C57BL/6 and C57BL/10 mice, whereas the strains A/J, BALB/c, DBA, CBA, C3H and SJL were poor responders (Kotani et al. 1990). Various TPO peptides were also found to be thyroiditogenic (Kotani et al. 1992, Ng,

Kung 2006, Hoshioka et al. 1993). Lastly, EAT has been reported in BALB/c mice after immunization with the extracellular domain of human TSHR expressed as a fusion protein with maltose-binding protein in *E.coli* (Costagliola et al. 1994).

For induction of EAT by the adoptive transfer protocol, splenocytes or lymph node cells (LNC) from mTg-primed mice, after *in vitro* activation with mTg for a 72 hour period, transfer thyroiditis to naïve recipient animals (Braley-Mullen et al. 1985). The recipient mice developed equivalent or higher levels of EAT compared to mice directly immunized with mTg in CFA or LPS (Braley-Mullen et al. 1985). Adoptive transfer of EAT was mediated by T cells because T lymphoblasts activated *in vitro* by monolayers of thyrocytes (Charreire, Michel-Bechet 1982), mTg-specific T cell clones (Romball, Weigle 1987) or T cell lines (Maron et al. 1983) were able to cause EAT in naïve mice. The adoptive transfer protocol has also been used successfully with Tg peptide-primed cells that were further activated *in vitro* with the immunizing peptide (Carayanniotis, Rao 1997).

A different type of EAT, called granulomatous EAT, was elicited by splenocytes from mTg-primed mice, further activated *in vitro* with mTg and an anti-interleukin-2 (IL-2) receptor Ab (Braley-Mullen et al. 1991). In this case, EAT was more severe than that adoptively transferred by lymphocytes cultured only with mTg. Granulomatous EAT was characterized by extensive follicular destruction, presence of multinucleated giant cells and proliferation of thyrocytes. Mice with granulomatous EAT also had higher mTg

specific-IgG responses compared to the group developing lymphocytic EAT (Braley-Mullen et al. 1991).

1.5.2. The role of MHC genes in murine models of EAT

To examine possible effects of H-2 haplotypes on susceptibility to EAT, Vladutiu et. al., challenged thirty three mouse strains, expressing 11 different H-2 haplotypes, with thyroid extract in CFA and monitored EAT development 4 weeks after the first immunization (Vladutiu, Rose 1971). Murine strains expressing the $H\text{-}2^{k}$ and $H\text{-}2^{s}$ haplotypes were characterized as excellent responders since they developed the highest pathology index and thyroid-specific Ab titers. Mice of the $H\text{-}2^{q}$ and $H\text{-}2^{a}$ type were reported to be good and fairly good responders respectively whereas mice with the $H\text{-}2^{b}$, $H\text{-}2^{d}$ and $H\text{-}2^{v}$ haplotypes were characterized as poor responders (Vladutiu, Rose 1971). This study showed that gene(s) within the H-2 locus influence the induction of EAT. Further work by Tomazic et al. mapped the major susceptibility gene(s) for thyroiditis between the K and A loci of the H-2 locus since intra-H-2 recombinant mice expressing the K^{k} and A^{k} alleles consistently developed thyroiditis at moderate to high levels compared to other recombinant mice (Tomazic et al. 1974). Finally, the generation of new suitable intra-H-2 recombinant strains of mice allowed identification of the *H-2A* gene as the principal gene controlling susceptibility to thyroiditis induction (Beisel et al. 1982). This finding was further supported by the fact that anti-H-2A mAb completely prevented EAT when administered around the time of immunization with Tg in CFA (Vladutiu, Steinman 1987). The crucial involvement of the H-2A locus in EAT induction

was also shown by introduction of the $A\alpha^k$ and $A\beta^k$ genes, expressed in good responder mice, to resistant (B10.M) or intermediate (B10.Q) responder mice, which led to a significant increase in the incidence and severity of thyroiditis compared to wild type (WT) control mice (Kong et al. 1997).

Other loci within the H-2 have been reported to affect the incidence and/or severity of thyroiditis. For example, B10.A and B10.AQR mice, differing at the K locus, developed thyroiditis at 52% vs. 70 % incidence, respectively. These data suggested that the presence of the K^k allele in B10.A mice vs. the presence of the K^q allele in B10.AQR mice lowered the incidence of the disease via an unknown mechanism (Beisel et al. 1982). In addition, point mutations in the K locus of the C57BL/6 mice were found to increase the incidence of thyroiditis from 20% in WT mice to 79%, after challenge with thyroid extract (Maron, Cohen 1979). However, these data were not confirmed by similar work performed in the same murine background by another group (Beisel et al. 1982).

In an analogous manner, the presence of certain D alleles - D^d, D^b and D^q – significantly reduced the levels of thyroid infiltration, as compared to the levels of disease in good responder mice (Kong et al. 1979). Also, the presence of the D^d and D^b alleles in strains expressing $H2K^k$ genes was associated with lower anti-Tg Ab responses than those observed in the $H\text{-}2^k$ progenitor strain (Tomazic et al. 1974). Later studies related the involvement of major histocompatibility complex (MHC) class I genes in EAT induction to production of cytotoxic T cells (Rose 2011), since anti-K^k and anti-D^k mAb completely

blocked the cytotoxic effects of mTg primed T cells on thyroid epithelial cells *in vitro* (Creemers et al. 1983).

The role of the E locus on EAT induction remains unclear since it has not been studied extensively. Previous work with intra-H-2 recombinant mice in the B10 background showed that the E^k molecule was necessary for EAT induction in mice of the H-2^k type after challenge with the Tg peptide (p2495-2511) in CFA (Chronopoulou, Carayanniotis 1993). However, the presence of the E^k molecule alone was not permissive for EAT induction, because mice expressing the resistant K^b, A^b, D^d alleles and the E^k molecule did not develop EAT. Findings from another study showed that introduction and expression of the $E\alpha^kE\beta^s$ molecule in B10.S mice led to a decrease in the severity of EAT compared to WT B10.S mice, which are E^-, after challenge with mTg in CFA or LPS (Kong et al. 1997). In the same study it was shown that the expression of a different E molecule, $E\alpha^kE\beta^d$ in B10.RQB3 mice did not affect EAT susceptibility, suggesting that different E alleles can have differential effects on EAT induction.

In humans, HT has been associated with the presence of certain human leukocyte antigen (HLA) proteins like HLA-DR4 (Jenkins et al. 1992, Thompson, Farid 1985), HLA-DR5 (Farid et al. 1981, Thomsen et al. 1983, Weissel et al. 1980) and HLA-DR3 (Golden et al. 2005, Tandon et al. 1990). Since conflicting data were reported regarding the association of HT and HLA-DR3 (Hasham, Tomer 2012), more studies have focused on this MHC class II molecule. To study its permissiveness to thyroiditis induction, DR3 transgenic

mice were generated in resistant B10.M mice in the absence of endogenous H-2A molecules (Kong et al. 1996). $DR3^+$ transgenic mice immunized with mTg or human Tg (hTg) developed significantly higher levels of mononuclear cell infiltration compared to $DR3^-$ mice that remained resistant to disease development (Kong et al. 1996). These data suggested that the DR3 molecule confers susceptibility to thyroiditis induction. The development of such HLA class II transgenic mice has allowed the examination of the role of HLA-DR and –DQ polymorphisms in susceptibility to EAT (Kong et al. 2007).

1.5.3. Pathogenic thyroglobulin epitopes in murine models of EAT

Immunologically, Tg has been reported to be the largest known autoantigen (van de Graaf et al. 2001). So far, 25 pathogenic T cell epitopes within Tg have been uncovered by different experimental approaches (**Figure 1.1**) (Carayanniotis 2007). The majority of the thyroidogenic peptides were identified by computer-based algorithms predicting putative MHC-binding Tg peptides (Caturegli et al. 1997, Chronopoulou, Carayanniotis 1992, Li, Carayanniotis 2006, Verginis et al. 2002, Flynn et al. 2004). Other approaches searched for the presence of common T cell epitopes between Tg and TPO (Hoshioka A 1993) or used T cell hybridomas to identify the minimal epitopes recognized by the T cell receptor (TCR) (Texier et al. 1992, Champion et al. 1991). More recently, studies using mass spectrometry identified Tg peptides eluted from HLA-DR-expressing cells from patients with Graves'disease (Muixi et al. 2008a, Muixi et al. 2008b). None of the known Tg peptides has been characterized as immunodominant according to criteria set by

Sercarz et al (Sercarz et al. 1993). The known pathogenic Tg peptides can be categorized into three groups based on their iodide content.

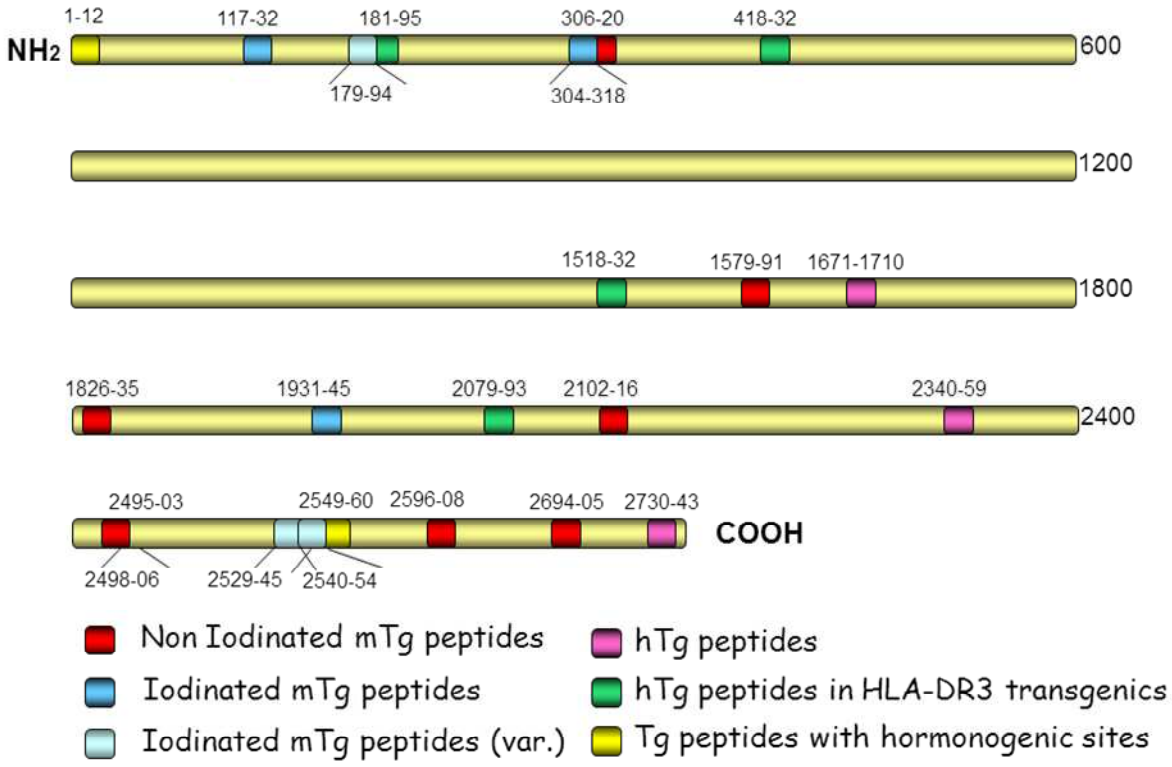

Figure 1.1

Relative positions of pathogenic Tg peptides prior to the initiation of the thesis work. Red: pathogenic peptides containing no iodotyrosyls; dark blue: peptides that are pathogenic only in their iodinated form; light blue: peptides in which the presence of iodine has variable effects on their pathogenicity; purple: pathogenic hTg-derived peptides; green: hTg-derived peptides found to be pathogenic in HLA-DR3 transgenic mice; gold: peptides containing hormonogenic sites. Numbers show a.a. coordinates without the leader sequence.

A) Pathogenic peptides containing hormonogenic sites.

The first pathogenic Tg peptide was identified in 1991 by Roitt's group (Champion et al. 1991). In that study, the A^k-binding peptide (a.a. p2551-59) containing T4 at position 2553 was reported to be recognized by two clonotypically distinct Tg autoreactive T cell hybridomas, CH9 and ADA2. The presence of T4 in the sequence was found to be critical since replacement of T4 by any other a.a. blocked the activation of both T cell hybridomas. Subsequent studies showed that the 12-mer peptide 2549-60 was able to induce proliferative (Hutchings et al. 1992) and cytolytic responses (Wan et al. 1998) in Tg-primed LNC and was able to cause lymphocytic (Hutchings et al. 1992) and granulomatous EAT (Braley-Mullen, Sharp 1997). The peptide p2549-60 was found to be pathogenic in the H-2^k CBA/J (Hutchings et al. 1992, Braley-Mullen, Sharp 1997, Kong et al. 1995), C57BR (Wan et al. 1997) as well as H-2^s A.SW and SJL (Wan et al. 1997) strains of mice. Interestingly, p2549-60 is the only Tg epitope so far that has been characterized as subdominant, since LNC from hTg- or mTg-primed mice proliferate in its presence *in vitro* (Hutchings et al. 1992, Kong et al. 1995, Wan et al. 1997). A second Tg peptide, p1-12, containing T4 at position 5, has also been reported to induce cytolytic responses (Wan et al. 1998) and cause mild lymphocytic thyroiditis (Kong et al. 1995). Not all epitopes containing T4 have a pathogenic potential, however. Peptides p2559-70 and p2737-48 with T4 at positions 2567 and 2746, respectively, were not immunogenic in the CBA/J background (Kong et al. 1995).

The finding that autoreactive T cells are activated by T4-containing peptides raised questions regarding the mode of interaction between the TCR and the peptide-MHC complex. To address whether the four iodine atoms within T4 are involved in T cell recognition, Kong's group generated an analog peptide of p2549-60 containing thyronine (T0) instead of T4 at position 2553. T0 retains the two-phenyl ring side chain of T4 without the four iodine atoms (Kong et al. 1995). Interestingly, the T0 containing peptide was also found to be immunopathogenic in CBA/J mice indicating that the presence of the four iodine atoms within T4 is not necessary for T cell activation at the polyclonal level. In contrast, in the same study it was shown that LNC from T4p2553-primed mice were unable to proliferate *in vitro* in the presence of the T0 analog peptide whereas they showed a strong proliferative capacity against the T4 analog. These data indicated the presence of distinct T cell clones responding only to the iodinated peptide. Similar findings at the clonal level have been reported by other groups showing that T4p2553-specific T cell hybridomas were activated *in vitro* only in the presence of the T4 containing peptide whereas the T0 analog was non stimulatory (Dawe et al. 1996, Dai et al. 2005). Data from these two studies also demonstrated that T4 was a critical residue for contact with the TCR, since the presence of a T4-specific Ab abrogated activation of the hybridoma.

B) Pathogenic peptides containing iodotyrosyl residues.

Previous work in our laboratory has shown that the three peptides I-p117 (a.a. 117-32), I-p304 (a.a. 304-18) and I-p1931 (a.a. 1931-45) were imunopathogenic in CBA/J mice

whereas the non-iodinated analogs p117, p304 and p1931 were not even immunogenic in the CBA/J background (Li, Carayanniotis 2006). The data from this study indicated that the presence of iodine in these peptides increased their pathogenic potential either by favoring their binding to the MHC or by activating T cell clones specific only for the iodinated epitopes. More studies, however, showed that the presence of an iodotyrosyl on a Tg peptide can affect its immunogenicity in multiple ways, i.e. it can either increase (p179, a.a. 179-94), decrease (p2540, a.a. 2540-54) or not affect (p2529, a.a. 2529-45) recognition by T cells at the polyclonal level (Li et al. 2007). Similarly, at the clonal level, a number of T cell hybridomas specific for the iodinated Tg peptide p179 or its non-iodinated analog required the iodine atom on the peptide for their activation. Other clones required the absence of the iodine atom, whereas activation of a third group of clones was independent of the iodine atom (Jiang et al. 2007).

C) Pathogenic non-iodinated peptides.

Several studies have reported the pathogenicity of non-iodinated Tg peptides (Carayanniotis 2007). The first identified peptide of this category was the 17-mer p2495 (a.a. 2495-2511), which was described to be thyroidogenic in the high responder murine strains SLJ, C3H and B10.BR (Chronopoulou, Carayanniotis 1992). Further work revealed that this epitope contained two overlapping determinants, the E^k and A^s-restricted 2496-2504 and the A^k-restricted 2499-2507 (Rao et al. 1994). The epitope 2496-2504 directly induced thyroiditis in C3H and SJL mice, whereas 2499-2507 caused weak EAT in both strains (Rao et al. 1994). In 1992, another group identified the 40 a.a.

peptide (F40D), (a.a.1671-1710), as the porcine Tg (pTg) fragment that could be recognized by a cytotoxic Tg-specific T cell hybridoma (Texier et al. 1992). In the same study it was shown that this peptide, located at the end of the second third of Tg, was able to cause EAT in CBA/J mice. In 1993, the epitope 2730-2743 was shown to be identical between hTg and human TPO (hTPO) and its pathogenicity was shown by adoptive transfers of Tg-primed splenocytes previously *in vitro* activated with this peptide to naïve mice. Recipients developed EAT whereas the control groups receiving phosphate buffered saline (PBS) or ovalbumin (OVA) had no thyroid infiltration (Hoshioka et al. 1993). In 1994, the peptide 2694-2711 was identified by algorithms as a putative T cell epitope and it was shown to be pathogenic in SJL mice (Carayanniotis et al. 1994). Further work showed that the major T cell determinant was spanning a.a. residues 2694-2705 (Rao, Carayanniotis 1997).

Searching for putative A^k binding peptides by Altuvia's algorithm (Altuvia et al. 1994) allowed identification of the following pathogenic peptides in CBA/J mice: 306-320, 1579-1591, 1826-1835, 2102-2116, 2596-2608 (Verginis et al. 2002) as well as 2529-2545 and 2540-2554 (Li et al. 2007). Following a similar algorithm-based approach, three HLA-DR3 binding peptides from hTg were found to be thyroidogenic in DR3 transgenic non-obese diabetic (NOD) mice; 2079-93 causes EAT consistently, whereas the peptides 1518-32, 181-195 cause only weak EAT (Flynn et al. 2004). A different methodology allowed identification of the peptide 2340-2359 from hTg as pathogenic in AKR/J (Karras et al. 2003) and DR3 transgenic C57LB/10 (Karras et al. 2005) mice by direct challenge. Initially, this epitope was found to be a target of Tg-specific B cells in

patients with Graves' disease. Recently, the peptide 409-423 from mTg was reported to be pathogenic in A^-E^+ C57BL/10 mice (Brown et al. 2008a) and the peptide 1677-1692 from mTg in A^-E^+ and A^+E^+ C57BL/10 mice (Brown et al. 2008b).

1.6. Antigen presentation in EAT

1.6.1. Dendritic cells

Most of the studies in autoimmune thyroiditis have focused on the role of dendritic cells (DC) and thyrocytes as antigen presenting cells (APC). DC reside intrathyroidally (Kabel et al. 1988, Klein, Wang 2004, Voorby et al. 1990), outside of the follicles and in close proximity to small capillaries (KAbel et al. 1988, Voorby et al. 1990). Little is known about the phenotype and function of resident DC, since their low frequency within the gland has hindered more extensive studies (Simons et al. 2000). So far, only one group has reported that the majority of intrathyroidal resident DC are characterized as $CD11c^{low}CD11b^+CD8\alpha^-$ cells, at a density of two cells per thyroid follicle (Klein, Wang 2004). Intrathyroidal DC were described to have an immature phenotype since there was no expression of the CD40 and CD80 proteins (Klein, Wang 2004), supporting findings from previous work that resident DC are in an immature state (Simons et al. 2000, Croizet et al. 2000). Regarding the function of intrathyroidal resident DC, it has been proposed that DC can inhibit the growth of thyrocytes via secretion of interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Simons PJ 1998), while thyrocytes can promote the immature state of DC via secretion of soluble factors (Croizet et al. 2001). Immunologically, it has been suggested that intrathyroidal DC constitutively express Tg

cryptic peptides on their MHC class II molecules, generated either after processing of Tg fragments or from processed Tg that had been transcytosed (Carayanniotis 2003). This is supported by the fact that activated Tg peptide specific $CD4^+$ T cells selectively home to the thyroid after direct challenge of mice with peptide in CFA or after adoptive transfer of activated cells to naïve mice. Under steady state conditions, presentation of these Tg peptides to naïve T cells should lead to peripheral tolerance either due to the absence of co-stimulatory signals on DC and/or low expression levels of peptides prohibiting T cell activation (Carayanniotis 2003).

Under inflammatory conditions, however, the presence of danger signals can induce the maturation and migration of intrathyroidal DC to the draining LN via the lymph. These mature DC subsets can initiate immune responses in the LN (Canning et al. 2003). This concept has been supported by the finding that necrotic thyrocytes were able to stimulate the maturation of bone marrow (BM) - derived DC *in vitro* as reported by the upregulation of MHC class II, CD80, CD86 and CD40 molecules (Li et al. 2006). Similar stimulatory effects on DC were not detected in the presence of healthy thyrocytes. In the same study, they also elicited Tg-specific Th1 and B cell responses as well as EAT (Li et al. 2006). Findings from spontaneous models of thyroiditis indicate that during the early stages of the disease there is a small increase in the number of DC intrathyroidally in the BB-DP rat (Voorby et al. 1990), the NOD mouse (Many et al. 1995) as well as in patients with HT, compared to those in the normal gland (Vasu et al. 2003).

The crucial role of DC in initiation of thyroiditis has also been shown in other studies. CBA/J mice, that do not develop spontaneously thyroiditis, developed the disease after injections of mTg-pulsed DC. The control groups receiving either PBS or DC-depleted splenocytes did not show any sign of infiltration or Tg specific-Ab in their serum (Knight et al. 1988). Similarly, DC pulsed with pTg stimulated proliferative Th1 responses of pTg-primed $CD4^+$ T cells from B10BR mice. Also, these animals injected subcutaneously (s.c.) with DC pulsed with pTg in the absence of adjuvant developed severe thyroiditis and anti-pTg IgG2a Abs, compared to animals receiving unpulsed DC (Watanabe et al. 1999).

Besides initiation of the disease, DC are also able to suppress the development of thyroiditis via activation of Tg-specific regulatory T cells (Treg). After treatment with TNF-α *in vitro*, BM-derived DC become semimature i.e. express high levels of MHC class II and co-stimulatory molecules, but they do not secrete proinflammatory cytokines (Menges et al. 2002). TNF-α treated DC were shown *in vivo* to activate interleukin-10 (IL-10)-producing Tg-specific cells that had a regulatory phenotype and suppressive function *in vitro*. Interestingly, repetitive injections of TNF-α treated DC pulsed with Tg were able to suppress development of thyroiditis in mice challenged with Tg in CFA. Similarly, $CD4^+CD25^+$ T cells isolated from mice injected with TNF-α treated DC pulsed with Tg, suppressed the incidence and severity of EAT (Verginis et al. 2005). In a similar manner, it was shown that in granulocyte-macrophage colony stimulating factor (GM-CSF) treated mice, only the $CD8\alpha^-$ DC subset acquired a tolerogenic phenotype and

function (Ganesh et al. 2009). *Ex vivo* analysis showed that this subset was able to increase the frequency of $Foxp3^+$ cells as well as IL-10-producing cells in mTg-primed T cells in the presence of mTg *in vitro*. $CD8\alpha^+$ DC from GM-CSF treated mice did not have such a capacity. Interestingly, adoptive transfers of $CD8\alpha^-$ DC from GM-CSF treated mice prevented EAT development in Tg-challenged mice (Ganesh et al. 2009). More studies revealed that the OX40L and Jagged1 signaling pathways have a crucial role in Treg expansion by GM-CSF BM-derived DC (Gopisetty et al. 2013).

1.6.2. Thyrocytes

Thyrocytes have also been reported to have an antigen presenting capacity within the thyroid gland. It has been shown that IFN-γ treated murine thyrocytes were able to generate and express the T4p2553 peptide on their surface as well as activate a T4p2553-specific hybridoma *in vitro* (Champion et al. 1991). Also, thyrocytes from patients with Graves' disease, which were MHC class II positive, were able to induce proliferative responses of the HA1.7 T cell clone in the presence of its ligand; the influenza strain A haemagglutinin peptide p20 (Londei et al. 1984). Under normal conditions, thyroid epithelial cells do not express MHC class II molecules (Voorby et al. 1990, Simons et al. 2000, Verma et al. 2000, Bonita et al. 2003), however, they become MHC class II positive under pathological conditions in humans, mice and rats (Voorby et al. 1990, Verma et al. 2000, Bonita et al. 2003, Hanafusa et al. 1983). This observation indicates that they do not have a primary role as APC in the initial events triggering thyroiditis. Also, work with transgenic mice expressing the MHC class II, $I\text{-}A^k$ gene constitutively on

thyrocytes showed that no SAT was detected in these animals, whereas there was only an 8% higher severity of the disease in transgenic vs. WT mice after immunization with Tg in CFA (Kimura et al. 2005, Li et al. 2004). These data clearly indicated that epithelial cells expressing MHC class II molecules alone are not sufficient to initiate autoimmunity. However, the fact that they can present Tg peptides to T cells indicates that thyrocytes may participate in amplification of the autoimmune response.

1.7. Spontaneous autoimmune thyroiditis

SAT has been reported to occur in chickens - the Cornell C Strain (CS) of White Leghorn (Cole et al. 1968) and the Obese Strain (OS) (Wick et al. 1974); rats – the Buffalo (Hajdu, Rona 1969) and the BB/W strain (Sternthal et al. 1981); dogs, monkeys and mice (Bigazzi, Rose 1975). In the following paragraphs, the murine models of SAT will be discussed in detail.

The NOD mouse is an animal model for insulin dependent diabetes mellitus. NOD mice, expressing the H-2^{g7} haplotype, (K^d, A^{g7}, E^0, D^b), usually develop diabetes at 60-80% incidence in female and 20-30% in male animals (Anderson, Bluestone 2005). Besides diabetes, NOD mice are prone to develop sialitis, prostatitis and SAT (Anderson, Bluestone 2005). Lymphocytic infiltration in the thyroid gland in NOD mice was initially reported to occur with 18.5% incidence (Asamoto et al. 1986). Later studies showed that the incidence of SAT in NOD mice varies among different colonies but mostly ranges from 5-25% (Many et al. 1995, Wicker et al. 1995, Damotte et al. 1997, Hutchings et al.

1999). Only one study has described very high incidence of SAT, reporting that 90% of 10 day-old NOD mice develop the disease in their colony (Bernard et al. 1992).

In regards to the presence of anti-Tg Abs in NOD mice developing SAT, one group has not detected them (Bernard 1992), whereas two other groups have reported a sporadic presence of anti-Tg Abs in older NOD mice (Damotte et al. 1997, Hutchings et al. 1999). The incidence and severity of SAT in NOD mice is comparable for female and male mice (Asamoto et al. 1986, Damotte et al. 1997, Bernard et al. 1992). Also, thyroiditis incidence was not reported to be significantly different in diabetic and non-diabetic NOD mice (Damotte et al. 1997, Bernard et al. 1992) supporting the concept that these two diseases are precipitated by dinstict mechanisms.

The NOD.H2^{h4} strain is one of several NOD MHC congenic strains that were generated by Dr. Linda Wicker's group investigating the role of the I-E MHC class II molecule in insulitis and diabetes development in the NOD background (Podolin et al. 1993). NOD.H2^{h4} mice, expressing the H-2^{h4} haplotype, K^k, A^k, E^0, D^b, do not become diabetic spontaneously or after treatment with cyclophosphamide (Podolin et al. 1993), but interestingly, they develop insulitis (Podolin et al. 1993), sialitis (Cihakova et al. 2009) and thyroiditis (Wicker et al. 1995, Kolypetri et al. 2010, Braley-Mullen et al. 1999, Burek et al. 2003, Weatherall et al. 1992). One group has reported a 50% incidence of thyroiditis in their colony (Wicker et al. 1995, Burek et al. 2003, Weatherall et al. 1992). A detailed kinetic analysis of SAT in our colony has shown disease onset around 15

weeks of age with SAT incidence increasing to 50% in 20-33-week old mice and reaching 100% in one year old animals. The severity of the disease increases over time but it can range from interstitial infiltration - infiltration index (I.I.)=1 - to extensive infiltration (I.I.=4) even in one year old animals (our unpublished data). Interestingly, similar findings have been reported by another group (Burek, Talor 2009).

1.7.1. The role of MHC genes in murine models of SAT

The role of individual MHC genes in the development of SAT has not been examined. However, the importance of the H-2 locus on the onset of SAT arises from the fact that NOD mice, H-2^{g7} haplotype, develop thyroiditis at a low incidence, ranging from 5-25% in different colonies (Many et al. 1995, Wicker et al. 1995, Damotte et al. 1997, Hutchings et al. 1999), whereas congenic NOD.H2^{h4} mice, H-2^{h4} haplotype, develop thyroiditis at 100% incidence by the age of one year old (Burek, Talor 2009). The high incidence of SAT in the NOD.H2^{h4} strain has been related to the combined presence of the A^k molecule with NOD non-MHC genes that are known to predispose to autoimmunity development (Wicker 1997). However, the NOD.H2^k strain, carrying the H-2^k haplotype, has been reported to develop SAT at 30.3% incidence by the age of almost 2 year old (Damotte et al. 1997). This indicates that additional genes within the H-2 locus, other than the A^k molecule, contribute to thyroiditis development. Such a gene could be *Ceat1*, which is tightly linked to the H-2 complex and has been reported to control thyroiditis induction in NOD mice (Boulard et al. 2002).

1.8. Links between iodine and thyroiditis development

Iodine is a trace element that has a central role in thyroid physiology since it acts as a substrate for the formation of the thyroid hormones, T3 and T4 and has a regulatory role in thyroid function (Saller et al. 1998). Iodine enters the human body through the dietary intake of milk and dairy products, fish, salt, seaweed, kelp, eggs and bread, drinking water or iodine-containing drugs, food preservatives and vitamin preparations (Roti, Uberti 2001).

After iodine enters the gastrointestinal tract, it is first converted to inorganic iodide and then it is completely absorbed. Following absorption, iodide circulates between the plasma, red blood cells, intraluminal fluids of the gastrointestinal tract and other extracellular compartments (Saller et al. 1998). The majority of inorganic iodide is stored within the thyroid gland (Saller et al. 1998). Besides the thyroid, iodide can also be found in the salivary glands, mammary glands, colon and placenta. Eventually, more than 90% of the iodide is excreted from the body through the kidneys (Cavalieri 1997). In the thyroid, epithelial cells actively take up inorganic iodide via NIS present on their basal membrane (Dai et al. 1996). Then, iodide is transferred to the apical surface of the cell due to its electrochemical gradient where iodide is oxidized by the enzyme TPO. Subsequently, TPO catalyzes the iodination of Tg and the thyroid hormones are generated (paragraph 1.5.1). Once T4 and T3 are released into the bloodstream, iodide is removed from MIT and DIT and either is reused or it enters the circulation (Dunn J.T., Dunn A.D. 2000, Cavalieri 1997).

The human thyroid gland requires 52 μg of iodine on a daily basis to remain in balance (Burgi 2010). According to the World Health Organization, an adult should receive up to 150 μg of iodine per day, i.e. 3x the daily required dose (WHO 2004), while the tolerable upper level of iodine intake has been set to 600 μg per day in the European Union and 1100 μg per day in the United States (Burgi 2010). The amount of iodine intake per day varies among countries with different dietary characteristics as well as among individuals within the same country (Rasmussen et al. 2009). For example, in populations consuming seaweed and fish, like the Japanese, the average dietary iodide can reach up to 2-3 mg/day whereas in the United States, Canada and parts of Europe, the intake is around 500 μg/day (WHO 2004). At an individual level, increased iodine supply has been related to chronic treatment with iodine-rich drugs like amiodarone, a medication used to treat cardiac arryhtmia, which releases 9 mg of iodine per day during its metabolism (Roti, Uberti 2001). Despite several studies (Roti, Uberti 2001, Burgi 2010) it still remains unclear whether high iodine ingestion is well tolerated over long time periods but reports have described a relation between increased incidence of autoimmune thyroiditis and high iodine intake in different countries like Brazil (Camargo et al. 2007), China (Teng et al. 2008), Japan (Konno et al. 1994), Argentina (Harach et al. 1985), United States (Weaver et al. 1966) and Switzerland (Hedinger 1981).

Similar observations have been described in animal models of autoimmune thyroiditis. An iodide-rich diet was reported to increase the incidence and severity of thyroidits in CS

chickens (Bagchi et al. 1985), Buffalo rats (Cohen, Weetman 1988), BB/W rats (Allen et al. 1986), NOD mice (Verma et al. 2000, Hutchings et al. 1999, Vecchiatti et al. 2013) and NOD.H2^{h4} mice (Hutchings et al. 1999, Kolypetri et al. 2010, Braley-Mullen et al. 1999, Burek, Talor 2009, Rasooly et al. 1996, Teng et al. 2009). Similar observations have been reported in iodide-deficient goitrous mice (Many et al. 1995, Mahmoud et al. 1986) and hamsters with hyperplastic goiters (Follis 1959). In contrast, high iodide intake did not induce SAT in SJL (Li, Carayanniotis 2007), CBA/J (Hutchings et al. 1999, Braley-Mullen et al. 1999, Li, Carayanniotis 2007) and Biozzi (Hutchings et al. 1999) mice. So far, the mechanism whereby iodide affects thyroiditis development remains unknown.

1.9. Iodide-accelerated SAT in NOD.H2^{h4} mice

In 1996, a study from Dr. Rose's laboratory showed that addition of 0.05% NaI in the drinking water of six to eight week old NOD.H2^{h4} mice for 8 weeks accelerated the incidence and severity of SAT (ISAT) compared to age-matched mice drinking normal water (Rasooly et al. 1996). The main charactestics of ISAT were: 1) mononuclear cell infiltration in the thyroid glands of fifteen out of 25 mice and 2) increased levels of anti-Tg-specific Abs in their sera. In the control group, the thyroid gland of only one out of 20 mice had a pathology index of 4, whereas none of the animals developed Tg-specific Abs. Interestingly, serum levels of the thyroid hormone T4 were similar in iodide treated and control mice, indicating that the iodide rich diet did not induce hypothyroidism in this strain as previously shown in SJL mice (Li, Carayanniotis 2007). This observation in

combination with the fact that the incidence of the disease in female and male mice was comparable, represent the two main differences between the human disease and this animal model of thyroiditis.

Notably, many groups have confirmed the findings described by Rasooly et. al. (Hutchings et al. 1999, Kolypetri et al. 2010, Braley-Mullen et al. 1999, Burek, Talor 2009, Teng et al. 2009, Oppenheim et al. 2003, Nagayama et al. 2007) and have tried to shed more light into the pathway leading to ISAT onset. A kinetic analysis of the disease in NOD.H2^{h4} mice has shown that the first signs of thyroid infiltration become evident after 2 weeks of NaI administration and become more prominent after prolonged iodide intake (Bonita et al. 2003, Kolypetri et al. 2010, Braley-Mullen et al. 1999). Immunohistochemical data have shown that CD4^{+} and α^{+} T cells are the first immune cells infiltrating the gland (Verma et al. 2000, Bonita et al. 2003, Yu et al. 2001). During this time point, few or no macrophages and no DC or natural killer (NK) cells have been detected in the thyroid lobes (Yu et al. 2001), whereas the presence of B cells has been reported by one group (Yu et al. 2001) but not confirmed by others (Bonita et al. 2003). After prolonged iodide administration, 4 to 8 weeks, an increased number of CD4^{+}, CD8^{+} T and B cells has been detected in the gland with CD4^{+} T cells being the predominant type of infiltrating cell (Bonita et al. 2003, Yu et al. 2001). At these stages of the disease macrophages, DC and NK cells are also present in the thyroid (Yu et al. 2001). Interestingly, the presence of such an inflammatory environment in the thyroid glands of iodide-fed mice is chronic and remains unchanged even after 23-24 weeks of treatment (Braley-Mullen et al. 1999, Teng et al. 2009). After 24 weeks of iodide administration,

when extensive infiltration is present in the thyroid, electron microscopy analysis has shown that thyrocytes have been described to have a high number of lysosomes containing lipofuscin granules, peroxisomes as well as mitochondrial swelling and dilatation of the rough endoplasmic reticulum compared to cells from control mice (Teng et al. 2009). Also, signs of apoptosis and necrosis have been reported in thyroid epithelial cells from iodide-fed animals at this stage (Teng et al. 2009).

In the serum, Tg-specific IgG Abs become detectable after 2-3 weeks of high iodide administration, reaching their maximum level 10-16 weeks later (Kolypetri et al. 2010, Braley-Mullen et al. 1999). The main subclasses of Tg-specific Abs have been identified as IgG2a, IgG2b and IgG1 (Hutchings et al. 1999, Braley-Mullen et al. 1999, Rasooly et al. 1996). Tg-specific IgM have also been detected in the sera of iodine-fed and control mice (Rasooly et al. 1996), but IgM binding to Tg appeared to be non-specific (Braley-Mullen et al. 1999). In older animals, 7 to 12 months old, IgG responses against mouse but not hTPO have been reported in iodide-treated and control mice by one group (Chen et al. 2010), whereas other studies were unable to detect them (Verma et al. 2000, Rasooly et al. 1996). The fact that TPO-specific Abs are detected at late stages of ISAT implies that TPO may not be the autoantigen triggering initiation of the autoimmune cascade in NOD.H2^{h4} mice.

1.9.1. The role of T cells in ISAT development

The involvement of T cells in ISAT development has been examined mostly by depletion studies (Verma et al. 2000, Hutchings et al. 1999, Braley-Mullen et al. 1999, Nagayama

et al. 2007, Yu et al. 2006). MAb-mediated depletion of $CD4^+$ cells during or at the end of the iodine administration period led to minimal or no thyroiditis development in NOD.$H2^{h4}$ mice compared to control mice receiving a rat IgG isotype control Ab. This supports the view that $CD4^+$ T cells are necessary for the development and progression of ISAT (Braley-Mullen et al. 1999). Depletion of $CD8^+$ T cells during the first weeks of iodine supplementation also decreased ISAT development, but when $CD8^+$ cells were depleted at the end of iodine supplementation period the treatment was ineffective. This suggests that $CD8^+$ T cells have an important role only in the initiation of ISAT (Verma et al. 2000, Braley-Mullen et al. 1999).

The involvement of another T cell subset, $CD4^+CD25^+$ Treg cells, in ISAT development remains unclear as shown by studies following different approaches (Nagayama et al. 2007, Yu et al. 2006, Ellis et al. 2013). Administration of anti-CD25 mAb to NOD.$H2^{h4}$ mice 4 days before initiation of iodide-rich diet led to increased severity of ISAT and higher production of anti-Tg Ab titers compared to untreated age-matched mice (Nagayama et al. 2007). These data indicated that $CD4^+CD25^+$ Treg cells can suppress ISAT development. In contrast, Yu's group reported that administration of anti-CD25 Ab to NOD.$H2^{h4}$ mice 10, 17 and 24 days after birth led to less severe ISAT after 8 weeks of NaI supplementation compared to mice receiving normal rat Ig (Yu et al. 2006). Also, elimination of Treg by thymectomy at 3 days of age did not affect ISAT incidence in iodine-fed mice compared to nonthymectomized control mice indicating that Treg cannot inhibit ISAT in NOD.$H2^{h4}$ mice (Yu et al. 2006). In another study, the same group

reported that $CD28^{-/-}$ $NOD.H2^{h4}$ mice which have reduced numbers of $CD4^{+}Foxp3^{+}$ cells in the spleen and cervical LN (CLN) compared to WT $NOD.H2^{h4}$ mice, developed significantly higher levels of ISAT compared to WT mice (Ellis et al. 2013). Also, adoptive transfer of $CD4^{+}Foxp3^{+}$ cells to $CD28^{-/-}$ $NOD.H2^{h4}$ mice reduced the severity of thyroiditis after 7 weeks of high iodide diet. Similar observations were not reported in $CD28^{-/-}$ mice receiving the same number of $CD4^{+}Foxp3^{-}$ cells or $CD28^{-/-}$ mice that did not receive any cells (Ellis et al. 2013). These data supported the view that Treg cells are involved and have the capacity to suppress ISAT in contrast to the opposite conclusions reached earlier by the same group. The involvement of Treg cells in ISAT progression was indicated by studies showing that splenocytes from iodide-fed animals for 8, 12 and 16 weeks had significantly lower percentages of $CD4^{+}CD25^{+}Foxp3^{+}$ cells and Foxp3 mRNA expression compared to mice receiving normal water (Xue et al. 2011).

NKT cells have also been implicated playing an effector role in ISAT development in $NOD.H2^{h4}$ mice. $NOD.H2^{h4}$ mice have been reported to contain unusually high numbers of NKT cells in their spleens, compared to age-matched BALB/c mice (Burek et al. 2003), and Tg-specific T cell lines generated from splenocytes of iodine-fed $NOD.H2^{h4}$ mice possess characteristics of NKT cells i.e. the presence of DX5 marker on their surface as well as the expression of Vα14Jα281 (Sharma et al. 2011). Stimulation of these cell lines with Tg was shown to be CD1d-restricted and led to production of interleukin-12 (IL-12), interleukin-4 (IL-4), IFN-γ, TNF-α and IL-10. Adoptive transfer of these T cell lines to iodide-pretreated $NOD.H2^{h4}$ recipients increased the production of Tg-

specific Abs and cellular infiltration in the thyroid glands, compared to the control group, indicating that NKT cells may have a pathogenic role in ISAT. However, transfer of the same T cell lines to NOD.H2^{h4} mice drinking normal water did not lead to thyroiditis development, suggesting that NKT cells cannot initiate ISAT in this strain. Their role is most likely exerted by cytokine release in the periphery, since transferred NKT cells did not migrate to the thyroid glands of NOD.H2^{h4} recipient mice (Sharma et al. 2011).

Another T cell subset that may be involved in ISAT development is Th17 cells (Horie et al. 2009). It has been shown that intrathyroidal expression of IL-17 as well as IFN-γ at the mRNA level was significantly higher in NOD.H2^{h4} mice after 4 and 8 weeks of iodide administration compared to control mice. Also, splenocytes from iodine-fed mice were reported to have an increased number of Th17 and Th1 cells compared to splenocytes from the control group, suggesting that iodine administration is related to an increased generation of both Th17 and Th1 cells. Interestingly, IL-17 knockout NOD.H2^{h4} mice were characterized by a significantly lower incidence and severity of infiltration as well as lower titers of Tg-specific IgG Ab compared to WT NOD.H2^{h4} mice after 8 weeks of iodine administration (Horie et al. 2009). However, another study by the same group, suggested that Th1 cells alone are able to induce thyroiditis (Horie et al. 2011). In this paper, they depleted the Treg cells from IL17$^{-/-}$ and IFNγR$^{-/-}$ NOD.H2^{h4} mice, which are known to be resistant to ISAT, and examined ISAT symptoms after 8 weeks on 0.15% NaI water. In NaI treated/Treg-depleted IL17 knock out mice, where only Th1 cells were present, ISAT symptoms were comparable to symptoms in WT mice

whereas, NaI treated/Treg-depleted IFNγR knock out mice had minimal ISAT. These data implied that Th1 cells alone are able to induce thyroiditis in this strain.

1.9.2. Antigens targeted by T cells in ISAT

NOD.H2^{h4} mice produce high titers of anti-Tg-specific Abs during early stages of ISAT suggesting that Tg-specific helper T cells have been previously activated. So far, however, studies have reported lack of or low detectable spontaneous T cell reactivity to mTg or to the A^k-binding Tg peptides I-p117, I-p179, I-p1931, I-p2529, I-p2540, p226, p1579, p1826 and p2026 using splenocytes after 21, 28, 42 and 56 days of iodide administration (Verma et al. 2000, Kolypetri et al. 2010, Braley-Mullen et al. 1999). Also, proliferative responses against Tg were not detected using thyroid draining CLN cells (CLNC) before ISAT onset - after 7 days of iodide administration (Kolypetri et al. 2010). Only one group so far has reported, but not shown, significant T cell responses to Tg in iodine-treated and untreated NOD.H2^{h4} mice (Rose et al. 1999, Rose et al. 1997). These observations outline a paradox between the abundance of Tg-specific IgG Abs and the low spontaneous T cell reactivity to Tg in NOD.H2^{h4} mice, in a similar manner to insulin in NOD mice (Kaufman et al. 2001).

To provide an explanation for this paradox, it was suggested that T cell responses are directed against a “modified form” of Tg. According to this premise, excess iodine intake may lead to formation of highly iodinated Tg (I-Tg) which is more immunopathogenic than Tg with normal iodine content (Verma et al. 2000, Burek, Rose 2008, Rose et al.

2002) as previously shown in the EAT model (Champion et al. 1987, Dai et al. 2002, Sundick et al. 1987). However, work from our laboratory showed that Tg preparations from NOD.H2^{h4} B cell deficient mice, designated as NOD.Kμ, drinking water with or without NaI, had comparable iodide content, suggesting that the NOD.H2^{h4} background does not favor generation of I-Tg *in vivo* after high iodide intake (Kolypetri et al. 2010). Furthermore, in the same study it was shown that *in vitro,* mildly iodinated Tg (24 iodine atoms/monomer) was not preferentially recognized by T or B cells compared to normal Tg (14 iodine atoms/monomer). This demonstrates that the high iodine content of Tg does not enhance its antigenicity in NOD.H2^{h4} mice and contrasts with previous results in the NOD.H2^{h4} model indicating that iodine can affect the antigenicity of Tg at the B and T cell level (Barin et al. 2005).

Significant proliferative T cell responses against hTPO were observed using splenocytes from iodide-fed mice as well as splenocytes from control 11 to 12 month old mice that had already developed spontaneous thyroid lesions (Verma et al. 2000). These data have not been confirmed by other groups so far. A concern about these findings is that the murine T cells may be cross-reactive to epitopes found only in hTPO and not present in murine TPO (mTPO). To exclude this possibility, the proliferation assays should be performed using mTPO.

1.9.3. The role of B cells in ISAT development

B cells have a crucial role during the onset and progression of ISAT in NOD.H2^{h4} mice. B cell-deficient NOD.H2^{h4} mice, designated as NOD.Kμ^{-}, develop minimal thyroid infiltration after 8 weeks of iodide supplementation, compared to WT NOD.H2^{h4} mice (Braley-Mullen, Yu 2000). In the same study, the presence of B cells was necessary during the first 4 weeks after birth for optimal ISAT development. B cell depletion using either an anti-IgM mAb during the first 3 weeks after birth (Braley-Mullen, Yu 2000) or an anti-CD20 – present on all mature B cells - mAb in 2-3 week old mice (Yu et al. 2012) resulted in minimal ISAT development compared to mice receiving an isotype control mAb.

A key role has also been attributed to B cells during the progression of ISAT in NOD.H2^{h4} mice (Yu et al. 2008). Seven to 10 week old mice receiving three injections of an anti-CD20 IgG2a mAb during an 8-week period of iodide-rich diet developed reduced ISAT symptoms compared to mice receiving an isotype control Ab (Yu et al. 2008). Interestingly, anti-CD20 IgG2a mAb was able to reduce ISAT symptoms even when its administration started 5 weeks after NaI treatment – i.e., in mice that had already developed ISAT symptoms at that time point (Yu et al. 2008). In this experiment, anti-CD20 treatment decreased the infiltration of B and T cells as well as the formation of T-B cell clusters in the thyroid gland compared to glands from mice receiving the isotype control mAb.

B cells may also have a pathogenic role after their infiltration into the thyroid gland since they have been reported to interact with $CD4^+$ T cells to form structures resembling germinal centers in the thyroid (Yu et al. 2001). A recent study focused on the intrathyroidal B cells in iodide-fed WT NOD.$H2^{h4}$ mice (Hong, Braley-Mullen 2014). This group reported that the majority of infiltrating B cells are follicular B cells expressing high levels of the costimulatory molecule CD80 and reduced levels of CD24, compared to splenic follicular B cells. The authors of this paper concluded that infiltrating B cells are more mature than splenic B cells. They also reported that thyroidal B cells were able to produce proinflammatory cytokines, TNF-a and IL-6, whereas they did not express the marker CD138 suggesting that they were not plasma cells.

Even though the B cell requirement for thyroiditis development has been well documented, the function of B cells during the different stages of the disease remains poorly understood. Data from studies so far support the idea that B cells may be required during early stages of life for optimal ISAT development, because they act as APC during the maturation of T cells (Braley-Mullen, Yu 2000). This concept was supported by the fact that when lethally irradiated mice were reconstituted with BM precursors from B cell deficient mice, minimal ISAT developed (Braley-Mullen, Yu 2000). However, transfer of BM precursors from B cell deficient mice and B cells in recipient mice resulted in ISAT development at comparable levels to iodide-fed WT mice. These data implied that B cells may be necessary as APC for the activation or maturation of autoreactive T cells early in life (Braley-Mullen, Yu 2000).

The production of IgG Abs by B cells seems to have a secondary role in ISAT pathogenesis. This premise was supported by the finding that passive transfer of NOD.H2^{h4} derived Tg-specific IgG to B cell-depleted NOD.H2^{h4} mice having an iodide-rich diet did not elicit disease, indicating that Tg-specific IgG production alone cannot lead to ISAT development. Similarly, irradiated adult NOD.Kμ^{null} mice, after reconstitution with B cells, were able to produce anti-Tg IgG but did not develop ISAT (Braley-Mullen, Yu 2000). The secondary role of antibodies in ISAT development was described in experiments with nitrophenyl (NPL) - receptor transgenic NOD.H2^{h4} mice (Yu et al. 2006). These transgenic mice, expressing only NPL-specific B cells that cannot secrete IgG, develop mild thyroiditis after NaI administration and anti-CD25 treatment was shown to exacerbate ISAT symptoms (Yu et al. 2006). Thyroids of anti-CD25 treated NPL transgenic mice were characterized by the presence of NPL-specific B cells, CD4+ and CD8+ T cells. These data suggested that: 1) transport of B cells to the thyroid is independent of their specificity and the production of IgG and 2) lymphocytic infiltration is independent of the production of Tg-specific IgG Abs. Thyroid-infiltrating B cells participate in the formation of germinal centers within the gland but their function still remains unknown (Hong, Braley-Mullen 2014).

1.10. Effects of iodide on thyroid epithelial cells

One of the mechanisms whereby iodide may initiate an autoimmune cascade in animals or humans is through thyrocyte death, i.e., the generation of an inflammatory

environment. Indeed high doses of iodide cause thyroid epithelial cell death in several animal species (Many et al. 1995, Teng et al. 2009, Bagchi et al. 1995, Li, Boyages 1994) or human thyroid follicles (Many et al. 1992) by *ex vivo* and *in vitro* studies. *Ex vivo* work examining the structure of the BB/W rat thyroid gland after 12 weeks on moderate (3×10^{-6} M) or high (3×10^{-3} M) iodide intake showed that thyrocytes had undergone ultrastructural changes compared to cells from control mice (Li, Boyages 1994). In thyrocytes from iodide-treated rats, swollen mitochondria as well as dilatation of rough ER were evident under electron microscopy. Also, accumulation of lipid droplets, condensation of chromatin, nuclear disintegration (karyolysis) and nuclear fragmentation (karyorrhexis) were observed in the iodide-treated group, whereas in the control group, thyrocytes were intact and only a few showed dilatation of the ER and mitochondrial swelling (Li, Boyages 1994). Similar observations have been reported in NOD.H2^{h4} mice after 8 to 24 weeks of iodide administration (1×10^{-5} – 2×10^{-3} M) (Teng et al. 2009), in the OS chickens 12 hours after an intraperitoneal injection of 250 μg of iodide (Bagchi et al. 1995) and NOD mice after 96 days of high iodide intake (10 mg/day) (Many et al. 1995). The interpretation of these findings has been that iodide may have a direct toxic effect on the thyrocytes of these animals.

In vitro work has further supported the concept that iodide can directly induce thyrocyte death. In several studies, primary thyroid cells or thyroid cell lines were cultured in the presence of different concentrations of iodide to estimate the rate of cell death. The range of iodide concentrations is wide, starting from 10^{-6} M (Lehmann et al. 2006, Langer et al.

2003), since the physiological iodine plasma levels are estimated to be 10^{-7} M for mice (Bernstein 2007, Taurog A. 1946) and humans (Many et al. 1992), to 10^{-2} M being the highest concentration (Yao et al. 2012, Golstein, Dumont 1996). Studies using relatively low iodide concentrations (10^{-6}-10^{-5} M) have been performed with porcine (Langer et al. 2003) and human (Lehmann et al. 2006) thyroid follicles. Specifically, intact porcine follicles were cultured with 2 x 10^{-6} M or 2 x 10^{-5} M potassium iodide for 8, 24 and 72 hours. The percentage of apoptotic cells was estimated as 4.18% ± 1.8% and 4.27 ± 2.68% for both doses after 8 hours of potassium iodide treatment whereas the incidence of apoptosis in untreated follicles was 1.14 ± 0.65%. After 24 and 72 hours, the number of apoptotic cells in the treated group remained unchanged. Interestingly, levels of necrosis in the culture were not affected by the presence of potassium iodide (1.13 ± 0.59). These data indicated that iodide was able to cause apoptosis and not necrosis in porcine thyroid follicles (Langer et al. 2003). Similar observations have been reported using human thyroid follicular cells (Lehmann et al. 2006). Incubation with 5 x 10^{-6} M and 1 x 10^{-5} M potassium iodide increased the rate of apoptosis to 4.3% and 5.8%, respectively, compared to the untreated group with an apoptotic rate of 3.38%. Despite the fact that the above studies were performed using low iodide concentrations, in an effort to model the *in vivo* conditions, the conclusions were weakened by the small differences detected in the apoptotic rate between the iodide-treated and untreated groups.

Studies using high iodide concentrations (10^{-4}-10^{-2} M) have been performed with a rat thyroid cell line, the FRTL-5 (Yao et al. 2012, Golstein, Dumont 1996), a human cell line

derived from fetal thyroid cells after infection by Simian virus 40, the TAD-2 (Vitale et al. 2000), as well as human thyroid follicles (Many et al. 1992) and primary dog thyrocytes (Golstein, Dumont 1996). Studies showed that TAD-2 cells cultured with 5 x 10^{-2} M KI for 24 or 48 hours underwent morphological changes, as observed under the light and fluorescence microscope and exposed the phospholipid phosphatidylserine on their cell surface, as detected by Annexin-V staining. DNA fragmentation had also occurred as demonstrated by gel electrophoresis (Vitale et al. 2000). All the above observations were signs of apoptotic cell death. Furthermore, KI treated TAD-2 cells were reported to produce high intracellular levels of reactive oxygen species (ROS) compared to KCl treated cells as well as lipid peroxides. Since the apoptotic pathway triggered by iodide in this cell line was not known, it was also examined whether iodide treatment affected the expression levels of p53, Bcl-2, Bcl-XL and Bax proteins – known to be involved in the mitochondrial apoptotic pathway. Interestingly, no change in the expression levels of these proteins was shown by Western blot analysis, suggesting that iodide-mediated apoptosis in TAD-2 cells does note require upregulation of these proteins (Vitale et al. 2000).

The iodide doses reportedly required to affect viability of FRLT-5 cells have been controversial. One study reported that their relative viability was significantly decreased when KI was added to the culture at 10^{-5}-10^{-2} M for 24 hours, as compared to that of untreated cells (Yao et al. 2012). As early as 2 hours after exposure to 10^{-4} or 10^{-2} M KI, there was a significant increase in the production of mitochondrial superoxide, but no

damage to the cell membrane. After 4 hours, a significant release of mitochondrial cytochrome c to the cytosol was reported in the iodide-treated group and damage of the cell membrane was indicated by increased lactate dehydrogenase activity in the supernatant. DNA fragmentation became evident after 24 hours of treatment with 10^{-4} or 10^{-2} M (Yao et al. 2012). Another study reported that FRTL-5 cells were susceptible to iodide-induced cell death only when 10^{-2} M of KI was used (Golstein, Dumont 1996), but specific pathways were not examined.

Effects of high iodide concentrations on the viability of cultured primary thyroid cells have been also examined. Human thyroid follicles incubated with 10^{-3} M NaI for 24 hours were reported to undergo approximately 10% necrosis whereas cells treated with 10^{-7} M NaI contained only 4% necrotic cells (Many et al. 1992). These data suggested that high iodide doses have a toxic effect on human thyroid cells. In contrast, primary dog thyrocytes incubated with 10^{-2} M NaI for 24 or 48 hours did not exhibit any signs of cell death compared to cells cultured only with medium (Golstein, Dumont 1996).

The majority of the studies described above have reported apoptosis as the most prevalent mode of iodide-induced cell death. The molecular pathway of iodide-induced apoptosis in thyrocytes has not yet been identified and it may be different between primary thyroid cells and cell lines. So far, however, a crucial step for the induction of iodide-induced apoptosis has been reported to be the organification of iodide by TPO since two inhibitors of TPO, methimazole and propylthiouracil, can abolish iodide-induced apoptosis in TAD-

2 cells and human thyroid follicles (Many et al. 1992, Vitale et al. 2000). Organified iodide can then be incorporated into lipids and proteins to form iodocompounds. Many studies have focused on the role of a certain iodolipid, 6-iodo-5-hydroxy-8, 11, 14-eicosatrienoic acid δ-lactone (δ-lactone) in apoptosis of thyrocytes (Gartner 2009). Δ-lactone has been isolated from porcine thyroid follicles (Dugrillon et al. 1990) as well as human thyroid tissue from patients with Grave's disease treated with high doses of iodide (Dugrillon et al. 1994). It has been shown that both iodine and δ-lactone induce apoptosis in porcine (Langer et al. 2003) as well as human thyroid follicles (Lehmann et al. 2006) and the thyroid carcinoma cell line B-CPAP (Gartner et al. 2010) through the mitochondrial pathway. Unfortunately, no more details about this signaling cascade in thyrocytes is known.

1.11. Other environmental factors affecting thyroiditis

Autoimmune thyroiditis is influenced by the interplay between genetics and environment and many exogenous factors contribute to the development of the disease (Saranac et al. 2011). For example, high iodine intake has been related to increased incidence and/or severity of thyroiditis in humans and in animal models (Ruwhof, Drexhage 2001) as discussed in detail in paragraph 1.8. Selenium deficiency has also been reported to contribute to the pathogenesis of thyroiditis via the generation of oxidative stress in thyrocytes (Kohrle et al. 2005). Environmental pollutants like polybrominated or polychlorinated biphenyls have also been related to oxidative stress generation in the thyroid gland and disease development (Saranac et al. 2011). Also, the presence of

infections agents like *Yersinia enterocolitica*, a Gram-negative coccobacillus (Strieder et al. 2003), hepatitis C virus (Testa et al. 2006) as well as drugs like amiodarone (Roti, Uberti 2001), interferon-α, interferon-β (Schuppert et al. 1997) and stress (Tsatsoulis 2006) have been linked to thyroiditis induction.

Recently, high salt diet (HSD) has been reported to be an environmental factor contributing to the development of autoimmunity (Wu et al. 2013, Kleinewietfeld et al. 2013). Specifically, it was shown that NaCl promotes the differentiation of Th17 cells *in vitro* and *in vivo* via the induction of the serum glucocorticoid kinase 1 (SGK1) followed by expression of the interleukin-23 receptor (IL-23R) (Wu et al. 2013, Kleinewietfeld et al. 2013). Also, these two studies reported that HSD can exacerbate experimental autoimmune encephalomyelitis (EAE) since C57BL/6 mice, challenged with the myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 in CFA, developed higher scores of the disease when they received a sodium-rich diet compared to the control group on normal diet. The increased severity of the disease was related to increased frequency of Th17 cells infiltrating the CNS. These studies raised the question of whether this mechanism would apply to other autoimmune diseases. In Chapter 6, we examined whether HS diet can exarcebate autoimmune thyroiditis using 3 animal models of the disease.

1.12. Co-authorship Statement

In **Chapter 3**, Hong Jiang, a former Master's student in our laboratory, identified the possible cathepsin B, D and L cleavage sites in mTg and prepared **Table 3.1**. She also used Altuvia's algorithm to identify the A^k-binding motifs close to cathepsin cleavage sites. In **Chapter 5**, Karen Carayanniotis, the research assistant in our laboratory, generated the NOD.H2^{h4}-derived T cell hybridoma clone KC1. Drs. Shofiur Rahman and Paris E. Georghiou from the Department of Chemistry, Memorial University of Newfoundland as well as Drs. Vassiliki Magafa and Paul Cordopatis from the Department of Pharmacy, University of Patras, were responsible for synthesis of F-moc L-T4. In **Chapter 6**, Dr. Bruce Van Vliet from the Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland, monitored the water intake over the 5 week period of the experiment and prepared **Figure 6.1A.** He also participated in design of the experiments, data analysis and provided comments and suggestions during manuscript preparation. Dr. Edward Randell from the Division of Laboratory Medicine, Faculty of Medicine, Memorial University of Newfoundland provided us the serum aldosterone kit.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

NOD.H2^{h4} mice, originally derived by Dr. L.Wicker (Merck Laboratories, Rahway, NJ), were kindly provided to us by Dr. H. Braley-Mullen (University of Missouri, Columbia, MO). The mice were bred and maintained under specific pathogen-free conditions in the animal facility of the Faculty of Medicine, Memorial University of Newfoundland. Both male and female mice were used and they were age- and sex-matched in each experiment. Female CBA/J and C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were used at 6-8 weeks of age. All experimental procedures were reviewed and approved by the Animal Care Committee at Memorial University of Newfoundland.

2.1.1. Generation of NOD.H2^{h4}.Foxp3^{gfp} mice.

NOD.H2^{h4}.Foxp3^{gfp} mice were generated in our colony by crossing male NOD.Foxp3^{gfp} mice, kindly provided by Dr. C.A. Piccirillo (McGill University, Montreal, Quebec) with female NOD.H2^{h4} mice. Female mice from the F1 progeny were backcrossed to male NOD.H2^{h4} mice. Offsprings of these matings (BC1 mice) were genotyped for the presence of Foxp3^{gfp} allele by PCR using the following set of primers: forward: 5'-A.A.GTTCATCTGCACCACCG-3', reverse: 5'-TCCTTGAAGAAGATGGTGCG-3'. Also, in the same reaction tube, amplification of an internal control gene was performed

with the following set of primers: forward: 5'-CTAGGCCAC AGAATTGAAAGATCT-3', reverse: 5'-GTAGGTGGAAATTCTAGCATCATCC-3'. PCR was performed for 35 cycles under the following conditions: 94° C for 30 seconds, 60° C for 1 minute and 72° C for 1 minute. PCR products were analyzed on a 1.8% agarose gel and had a size of 173 bp for the gfp product and 324 bp for the internal control product. BC1 mice were also genotyped for expression of the H-2^{h4} or H-2^{g7} haplotype by staining peripheral blood cells with Phycoerythrin (PE)-conjugated K^k, K^d and isotype control mAb (BD Pharmingen, Mississauga, ON, Canada) and analyzing by flow cytometry. To exclude the possibility of intra-MHC recombination, peripheral blood cells from K^k homozygous mice were stained with a Fluorescein isothiocyanate (FITC)-conjugated I-A^d mAb (BD Pharmingen), cross-reactive to I-A^{g7} but not I-A^k molecule and were found to be negative for the I-A^{g7} expression. BC1 mice, homozygous for the K^k allele and expressing the Foxp3^{gfp} allele were intercrossed. Female mice homozygous for the Foxp3^{gfp} allele were identified by PCR analysis using the primers: forward 5'-GCCTCTGACAAGAACCCAATG-3' and reverse 5'-CTGGTCCCTAGAAGTTCTGAG-3'. Amplification was performed at 94° C for 30 seconds, 65° C for 1 minute and 72° C for 1 minute for 35 cycles. The PCR products had a size of 133 bp for the WT Foxp3 allele and 852 bp for the Foxp3^{gfp} allele.

2.1.2. Special diet

NOD.H2^{h4} and NOD.H2^{h4}.Foxp3^{gfp} mice received 0.05% NaI water starting at 6-8 weeks of age for the indicated time period. CBA/J and C57BL/6J mice were on a special diet as

described by Kleinewietfeld et al. (Kleinewietfeld et al. 2013). The high salt diet groups received a combination of salt-enriched chow (4% NaCl Teklad TD.92034) and water supplemented with 1% NaCl. Control mice received a regular sodium diet (0.49% NaCl, Teklad TD.96208) and deionized distilled water (autoclaved before distribution) *ad libitum*. The commercial rodent chow had a natural ingredient base containing wheat, corn, soy and alfalfa; (w/w) 19% protein; 48% carbohydrates; 5.2% fat; 0.9% Ca; 0.8% K. Water intake was monitored weekly and is expressed as grams consumed per mouse per day.

2.2. Antigens and peptides

Tg was purified from frozen thyroid glands of ICR mice (Bioproducts for Science, Indianapolis, IN) or CD1 mice (Charles River, Quebec, Canada) by gel filtration. Briefly, thyroid glands were homogenized in PBS buffer, pH=7, containing 10^{-6} M leupeptin, 10^{-6} M pepstatin and 10^{-3} M phenylmethanesulfonylfluoride (Sigma Aldrich, St. Louis, MO). Extracts were centrifuged 3x at 16,000 g for 10 minutes and supernatant was loaded to a Sepharose CL-4B (Amersham Biosciences AB, Uppsala, Sweden) column. Fractions were collected and their optical density was determined at λ=280 nm by spectrophotometry (Life Science UV/Vis spectrophotometer, Beckman Coulter, Du® 730). Based on the elution profile of the supernatant, the fractions forming the second peak of the chromatogram (**Figure 2.1**) were pooled, dialyzed 3x in distilled water concentrated to 1-2 mg/ml (Amicon, Millipore Corporation, Bedford, USA), lyophilized in Speedvac and stored at -20° C. OVA was purchased from Sigma. The Tg peptides,

(557-568), FLVFLQRAVSVP, p557; (771-781), QNGDGQELTPA, p771; (1822-1832), SDFPGDMATEL, p1822; (2369-2380), DVASIHLLISRP, p2369; (2439-2450), NILNDAQTKLLA, p2439; were synthesized by Biosynthesis Inc. (Lewisville, Texas) and were used at >85% purity. F-moc L-T4 was produced as previously described (Kong et al. 1995, Adamczyk et al. 1994). The 12 mer Tg peptide T4p2553 (a.a. 2549-2560), STDD(T4)ASFSRAL was synthesized at the University of Patras, Greece, and at Biosynthesis, Inc (Lewisville, Texas, USA) at >90% purity. The 19mer OVA peptide (322-340), CISQAVHAAHAEINEAGRY, was synthesized at the Alberta Institute (Edmonton, Alberta, Canada) and the purity was 70%. All peptides were blocked with an acetyl group at the N-terminus and with an amide group at the C-terminus.

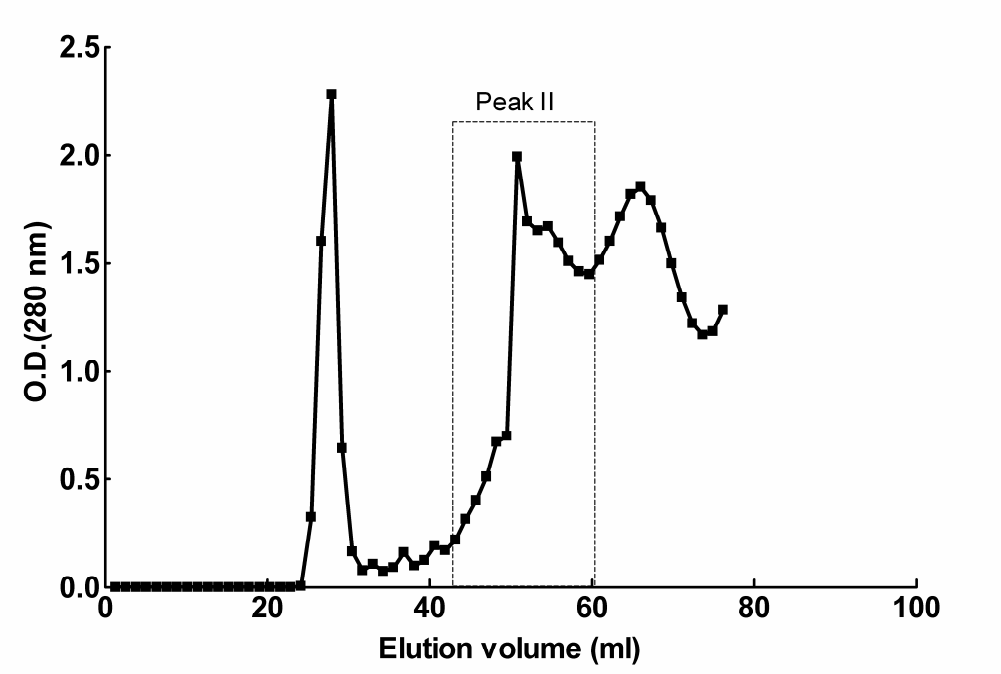

Figure 2.1

Thyroid glands from 20 ICR mice were homogenized in PBS buffer, pH=7, as described in Materials and Methods. The thyroid extract was passed through a Sepharose CL-4B column and its elution profile is shown above. The box indicates the elution volume of free Tg. The fractions within the box were pooled, dialyzed in distilled water, concentrated to 1-2 mg/ml and lyophilized. Samples were kept at -20 ^{o}C until use.

2.3. Cell lines and tissue culture

The TA3 cell line, kindly gifted by Dr. L. Glimcher (Harvard University, Cambridge, MA) and provided by Dr. T. Watts (University of Toronto, Ontario, Canada), was produced by fusion of B cells from CAF1 mice with the M12.4.1 BALB/c B lymphoma (Allen et al. 1985). The IL-2 dependent CTLL-2 cell line, purchased from the American Type Culture collection, was cultured in medium supplemented with 10% supernatant from Concanavalin A-activated rat spleen cells. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Invitrogen, Grand Island, NY), supplemented with 10% Fetal Calf serum (FCS) (PAA Laboratories, Ontario, Canada), 20 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies) and 5 x 10^{-5} M 2-mercaptoethanol (Sigma). The NOD.H2^{h4}-derived T4p2553-specific T cell hybridoma KC1 was generated following a modified procedure (Perkins et al. 1991). Briefly, NOD.H2^{h4} mice were immunized with 100 nmol T4p2553 peptide in CFA emulsion. Nine days later, draining LN were collected and single cell suspensions were prepared in DMEM supplemented with 10% FCS. Cells were cultured in the presence of 20 μM T4p2553 for 4 days and subsequently were fused with BW5147$\alpha^-\beta^-$ cells, kindly provided by P. Marrack (National Jewish Medical and Research Center, Denver, CO) in a 1:1 ratio in the presence of polyethylene glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany). After this fusion step, cells were cultured in the presence of hypoxanthine-aminopterin-thymidine containing DMEM supplemented with 20% FCS for 7-21 days followed by a 10-day culture in hypoxanthine-thymidine containing DMEM supplemented with 20% FCS. Cells that

survived the selection procedure were cultured in DMEM supplemented with 10% FCS and their specificity was tested as described below. T4p2553-specific T cell hybridomas were cloned by limiting dilution at 0.3 cells/well using red blood cells (RBC) as feeder cells.

2.3.1. Primary thyrocyte culture

Thyroid cells were isolated and cultured according to a modified method (Jeker et al. 1999). Thyroid lobes from mice were aseptically removed from the trachea, cut into small fragments, teased apart and material from three glands was placed in 1 ml DMEM digestion medium (Gibco, Life Technologies, Grand Island, NY, USA) containing 1 unit /ml of collagenase type I (Sigma-Aldrich, St. Louis, MO) and 1.2 units/ml of dispase I (Sigma). Enzymatic digestion was performed at 37° C for 45 minutes followed by vigorous pipetting. The cells were then washed twice and were re-suspended in F-12 Nutrient Mixture (HAM) culture medium (Invitrogen) supplemented with BD^{TM} Nu-Serum IV Replacements (BD Biosciences, Bedford, MA, USA) (diluted 2.5 x), glycyl-L-histidyl-L-lysine acetate salt (2 ng/ml final), somatostatin (10 ng/ml), TSH (10 mU/ml) and NaI (1 μM) (all from Sigma).

To ascertain purity, thyrocytes were cultured as described above in 8-well chamber slides (Nalge Nunc International, Rochester, NY, USA) using material from three NOD.H2^{h4} thyroid glands per well. On day 10, cells were fixed with acetone for 10 min at -20° C, washed, and subsequently were placed in PBS containing 10% FCS for 20 min at room temperature (blocking step). Then, cells were incubated with Tg-specific antisera

from iodide-fed NOD.H2^{h4} mice or antisera from control CBA/J mice (1:20 dilution) for 1 hour. The cells were then washed 3x and incubated with a secondary FITC-labeled goat anti-mouse IgG (Jackson Immunoresearch LAboratories, West Grove, PA, USA) for 30 min. Cells were again washed 3x and one drop of VECTASHIELD Hard-Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was added to each well. Fluorescent cells were viewed using a Carl Zeiss AxioImager Z.1 microscope.

2.4. T cell hybridoma activation / inhibition assay

Specificity of T cell hybridoma KC1 was examined by an activation assay in which 10^5 TA3 cells and 10^5 T cell hybridomas were cultured in wells of a 96-well plate in the presence of titrated amounts of T4p2553 and Tg (starting concentration 100 nM). After 24 hours, supernatant from wells was removed and its IL-2 content was assessed upon incubation with the IL-2 dependent CTLL cells. CTLL proliferation was measured by [^{3}H] thymidine incorporation. MHC restriction of KC1 was examined by an inhibition assay. 10^5 TA3 cells and 10^5 T cell hybridomas were cultured in the presence of 25 nM T4p2553 and 10 μg/ml of anti-A^k or anti-influenza A nucleoprotein (NP) mAb, purified as previously described (Verginis et al. 2002), as inhibitors. Percent of inhibition was calculated as follows: [1-(cpm in the presence of mAb)/(cpm in the absence of mAb)] x 100.

2.5. Immunizations and T cell proliferation assays

CBA/J and NOD.H2^{h4} mice were challenged s.c. at the base of the tail and the dorsal side of the neck with 100 nmol of peptide or 100 μg of Tg in 100 μl CFA emulsion (with

Mycobacterium butyricum; Difco LAboratories). After nine days, the inguinal, branchial and axillary LN were isolated and single cell suspensions were prepared in DMEM supplemented with 10% FCS, 20 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 x 10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO). After centrifugation and washing, 4 x 10^5 cells/well were cultured with or without titrated amounts of peptide (0.5-25 μM), Tg or OVA (1 μM) in flat-bottom 96-well plates and were incubated for 4 days at 37° C in a 10% CO_2, 90% air-humified incubator. Eighteen hours prior to harvesting, 1 μCi of [^{3}H] - thymidine (6.7 mCi/mmol; Perkin Elmer, Boston, USA) was added to each well. The cells were harvested using a Classic Cell Harvester (Skatron Instruments) and radioactivity was measured using the LS6500 Multi-Purpose Scintillation Counter. Stimulation index (S.I.) was defined as: cpm in the presence of Ag/cpm in the absence of Ag.

2.6. Spontaneous T cell proliferation assays

For assessment of spontaneous T cell proliferation, CLN and spleens were removed from NOD.H2^{h4} mice after 7, 11 and 15 days of iodide treatment. Single cell suspensions were prepared in HL-1 medium (Lonza, Walkersville, MD, USA) supplemented with 100 U/ml penicillin/streptomycin, 2 mM glutamine and 5 x 10^{-5} M 2-ME and were cultured at 4 x 10^5 cells/well in the presence of 10 μg/ml T4p2553, 20 μg/ml Tg, 20 μg/ml OVA for 3 days at 37° C, in a 5% CO_2 incubator. During the last eighteen hours, 1 μCi [^{3}H] thymidine (Perkin Elmer, Boston, MA) was added to each well. Then, cells were harvested and radioactivity was measured as described above.

2.7. Detection of IgG responses by enzyme-linked immunosorbent assay (ELISA)

Serum IgG antibodies specific for Tg or Tg peptides were detected by a two-step ELISA, as previously described (Kolypetri et al. 2010). Briefly, wells of polyvinylchloride plates were coated overnight with 10 μg/ml Tg or 4 μg/ml Tg peptide in carbonate buffer, pH= 9.6 and blocked with 0.1% bovine serum albumin in PBS for 1 hour. Serum samples, diluted in PBS/Tween 20 containing 0.1% bovine serum albumin, were added to wells for 1 hour and specific ab binding was assessed with an alkaline phosphatase-conjugated goat anti-mouse IgG Ab (Jackson Immunoresearch LAboratories, Inc., West Grove, Pennsylvania). After 20 minutes, conversion of the p-nitrophenyl phosphate substrate (Sigma) into the p-nitrophenol product was determined by light absorption at 405 nm using a Vmax plate reader (Molecular Devices, Sunnyvale, CA).

2.7.1. Detection of cytokines in the culture supernatant

The presence of IL-2, IFN-γ and IL-4 in the culture supernatant of LNC after 48 hours was assessed by sandwich ELISA via extrapolation from standard curves generated using known amounts of murine recombinant IL-2, IFN-γ and IL-4 (BD Biosciences, San Diego, CA), according to the manufacturer's instructions. Light absorbance was measured using a Vmax plate reader at 450 nm. The detection limits were 4 pg/ml for IL-2, 40 pg/ml for IFN-γ and 8 pg/ml for IL-4.

2.7.2. Detection of aldosterone in mouse serum.

Serum aldosterone was assessed by RIA (Coat-a-count Aldosterone kit TKAL1, Siemens) according to the manufacturer's instructions.

2.8. EAT induction and histological assessment

EAT was induced in mice either directly or upon adoptive transfers of *in vitro* activated cells. CBA/J and NOD.H2^{h4} mice were immunized s.c. with 100 nmol of peptide emulsified in CFA and three weeks later were challenged with 50 nmol of the same peptide in incomplete Freund's adjuvant (IFA). C57BL/6J mice were immunized s.c. with 100 μg of Tg in 100 μl of CFA and three weeks later were boosted with 50 μg of Tg in IFA. EAT was assessed two weeks after the last challenge. Induction of EAT by adoptive transfer was conducted as previously described (Rao et al. 1999). Briefly, LNC isolated from donor CBA/J mice, previously challenged s.c. with 100 nmol of peptide in CFA, were incubated for 72 hours in the presence of 25 μM of the appropriate peptide. After harvesting and washing, 2 x 10^7 cells in 200 μl of PBS were administered i.p. into syngeneic recipients and EAT was evaluated 14 days later. Fixation, embedding in methacrylate and sectioning of thyroids were performed as previously described (Rao et al. 1999). The sections were stained with hematoxylin and eosin and were scored for thyroid pathology as follows: 0, no infiltration; 1, small interstitial infiltration; 2, one or two foci of inflammatory cells; 3, diffuse infiltration of 10-40% of total area; 4, extensive infiltration, 40-80% of total area and 5, extensive infiltration over 80% of total area.

2.9. Microscopic detection of apoptosis

Cells from 10 thyroid glands were added into 18 wells of black 96-well plates (Costar #3603, Corning Incorporated, NY, USA**)** and were cultured for 10 days at 37° C, 5% CO_2. Medium was changed one day later and then every third day. On day 9, the plates were centrifuged and cells were washed 2x to remove non-adherent, floating cells. Then, various concentrations of NaI (Sigma) or 6 μM camptothecin (Sigma) were added to the cells for 24 hours. Apoptosis was assessed by the FAM-FLICA^{TM} *in vitro* Poly Caspases kit (Immunochemistry Technologies, Bloomington, MN, USA), which utilizes a 3-aminoacid sequence (VAD) sandwiched between carboxyfluorescein (FAM) and fluoromethylketone (FMK). The FAM-VAD-FMK reagent penetrates the cell membrane and binds irreversibly to activated caspases 1, 3, 4, 5, 6, 7, 8 and 9. During the last five minutes, propidium iodide and Hoechst 33342 were also added to the wells. After extensive washing (10x), live cells were observed under an inverted Carl Zeiss AxioObserver A.1 microscope. Images were captured using a Zeiss AxioCam MRM3 camera with Zeiss Axiovision 4.8 Software. At least 300 cells per group were counted and analyzed by the ImageJ 1.41 Software (NIH, USA).

2.10. Expression analysis of apoptosis and oxidative-stress related genes by Real-Time PCR

The expression of 168 genes involved in apoptotic and oxidative stress-related pathways was measured by the mouse Apoptosis and Oxidative stress PCR arrays (Qiagen Sciences, Maryland, USA). Thyroid glands from 80 NOD.H2^{h4} and 80 CBA/J mice were

isolated and cultured as described above. On day 9, thyrocytes were incubated with or without iodide (4 x 10^{-6} M final concentration) for 24 hours. Then, the supernatant of cultured thyrocytes was removed and the cells were lysed by the addition of Trizol (InVitrogen). The aqueous phase was mixed with an equal volume of 70% ethanol and subsequently the sample was transferred to an RNeasy spin column (Qiagen). The RNA was purified following on-column DNase treatment (RNase-free DNase set, Qiagen) according to the manufacturer's instructions. RNA purity was quantified by spectrophotometry at 260/230 nm and 260/280 nm ratios. The cDNA was transcribed from 1 µg total RNA per group using the RT^2 First Strand Kit (Qiagen,) in a total volume of 222 µl. Next, 102 µl of cDNA (equivalent to 0.5 µg RNA) were mixed with 1350 µl RT^2 SYBR Green Mastermix and RNase-free water in a total volume of 2700 µl. Twenty-five µl of this PCR components mix were added to each well of the RT^2 Profiler PCR Array and quantitative PCR was performed using a StepOnePlus Applied Biosystems cycler. The cycling conditions were 95^0 C for 10 min for activation of HotStart DNA Taq Polymerase followed by 40 cycles of denaturation at 95^0 C for 15 s and annealing at 60^0 C for 60 s. A dissociation (melting) curve analysis was performed to verify PCR specificity. Each 96-well plate contained primers for 84 pathway-related genes and 5 housekeeping genes whereas 7 wells contained reverse-transcription controls, positive PCR controls and genomic DNA contamination control. For each gene of interest, the difference (ΔC_T) between its C_T value and the average C_T value of the 5 housekeeping control genes was calculated. For each pair-wise set of samples to be compared, the difference in ΔC_T values was calculated as: $\Delta\Delta C_T = \Delta C_T$ (exp) - ΔC_T

(control) and the fold-change of target gene expression was calculated as $2^{(-\Delta\Delta CT)}$. Fold change values of <1 are expressed as negative inverse of fold change.

2.11. Purification of DC from spleen and CLN

To obtain DC, spleens and/or CLN were digested with Liberase TM research grade (1.67 Wünsch units/ml RPMI) (Roche Applied Science, Quebec, Canada) for 30 minutes at 37° C. After a gentle pipetting, cell suspensions were passed through a nylon mesh to remove undigested material, washed, and then incubated with PBS supplemented with 5% FCS and 5 mM EDTA, pH 7.2, for 5 minutes to disrupt DC/T cell complexes. After an additional wash, splenic cells were treated with RBC lysing buffer (Sigma) while CLNC suspensions were passed through Lympholyte M (Cedarlane, Burlington, ON, Canada) to remove RBC and debris. The cells were resuspended in PBS containing 0.5% BSA and 2 mM EDTA and DC were positively selected using anti-mouse CD11c-coated magnetic beads and MACS columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The purity of the $CD11c^+$ population was >93%, as confirmed by flow cytometry.

2.12. Purification of thyroid infiltrating lymphocytes.

Intrathyroidal lymphocytes (IL) were obtained from 15 NOD.$H2^{h4}$ mice drinking 0.05% NaI water for 35 days. Thyroids were treated with 0.28 U/ml Liberase TM research grade (Roche Applied Science) in serum-free RPMI -1640 medium for 45 minutes at 37° C. Undigested material was mechanically disrupted by pipetting and passing through a 35-

μm diameter nylon cell strainer (BD Discovery LAbware, Mississauga, ON, Canada). Lymphocytes were separated using Lympholyte M.

2.13. Flow cytometry and intracellular cytokine staining.

$CD4^+$ cells were purified from CLNC and/or spleens using a mouse $CD4^+$ T cell enrichment kit (StemCell Technologies, Vancouver, Canada). Subsequently, they were cultured at 2 $x10^5$ cells/well in HL-1 medium with splenic DC (4 x 10^4 cells/ well) and antigen in 96 flat-bottom well plates for 4 days. During the last 6 hours, 5 μg/ml of Brefeldin A (Sigma) were added to the wells. All staining steps were performed at 4^o C. FcR were blocked with anti-mouse CD16/32, (clone 93; eBiosciences, San Diego, CA) for 15 min and subsequently incubated with an FITC-conjugated anti-CD4 mAb or isotype control (BD Biosciences, Mississauga, Ontario, Canada) for 30 min. After three washing steps, cells were fixed in 2% paraformaldehyde and permeabilized with 0.5% saponin in PBS + 2% FCS. Then, staining using a PE-conjugated anti-mouse IFN-γ and the isotype control mAb was performed (BD Biosciences) for 30 minutes at 4° C.

2.14. Statistical analysis

Statistical significance was assessed by the non-parametric Mann-Whitney test or by a one-tailed unpaired Student's t-test using the GraphPad Prism 4 Software.

CHAPTER 3

Identification of pathogenic T cell epitopes near cathepsin cleavage sites in thyroglobulin

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3.1. Abstract

EAT, induced in mice after challenge with Tg, is known to be under the genetic control of the *H2A*^{k} locus. Because cathepsins are known to influence proteolytic processing of Tg *in vivo*, we examined in this study whether putative H2A^k-binding Tg epitopes, located near cathepsin cleavage sites within mTg, have immunopathogenic properties. Cathepsin L, B, and D cleavage sites in mTg were predicted based on homology with known cathepsin cleavage sites in rabbit Tg. We used an algorithm-based approach to identify H2A^k - binding motifs within 20-a.a. residue segments adjacent to cathepsin cleavage sites, and five 12-mer peptides encompassing these sequences were synthesized. Two of them, p2369 (a.a. 2369-2380) and p2439 (a.a. 2439-2450) were immunogenic, eliciting significant proliferative T cell responses using LNC from peptide-primed mice and production of IL-2 and IFN-γ in recall assays *in vitro*. Both peptides induced EAT upon direct challenge of CBA/J mice with peptide in CFA and by adoptive transfer of peptide-primed LNC into naïve recipient hosts, but neither peptide was characterized as dominant.

3.2. Introduction

EAT, a model of HT in humans, is induced in mice after challenge with Tg in adjuvant (Rose et al. 1971, Esquivel et al. 1977). Successful induction of EAT is characterized by infiltration of mononuclear cells in the thyroid gland, follicular cell destruction and production of Tg–specific Abs (Weetman, McGregor 1994). Early studies have delineated the importance of the MHC region in EAT induction (Vladutiu, Rose 1971) attributing the genetic control of the disease to the $H2A^k$ locus in $H2^k$ mice (Beisel et al. 1982, Kong et al. 1997). EAT is considered to be a T-cell mediated disease (Braley-Mullen et al. 1985, Romball, Weigle 1987, Maron et al. 1983, Vladutiu, Rose 1975) and $CD4^+$ T cells play a pivotal role in the initiation and development of the disease (Flynn et al. 1989).

Tg, the most abundant thyroid protein, is produced by thyrocytes and then is exported and stored in the follicular lumen (Dunn J.T., Dunn A.D. 2000). Since its discovery as an autoantigen in 1956 (Roitt I.M. et al. 1956), 25 Tg pathogenic epitopes have been identified using a variety of approaches (Carayanniotis 2007). A common strategy involved the use of computer algorithms to delineate putative MHC-binding Tg peptides (Caturegli et al. 1997, Chronopoulou, Carayanniotis 1992, Li, Carayanniotis 2006, Verginis et al. 2002, Flynn et al. 2004) whereas other methods employed cloned T-cell hybridomas as screening tools (Texier et al. 1992, Champion et al. 1991), elution of human MHC-bound peptides (Muixi et al. 2008a, Muixi et al. 2008b) or focused on

searching for T-cell epitopes shared between Tg and TPO (Hoshioka et al. 1993). All known Tg pathogenic peptides have fallen under three categories: a) iodinated peptides containing iodotyrosyls; b) peptides encompassing hormonogenic sites; and c) non-iodinated peptides scattered throughout the Tg molecule (Carayanniotis 2007). Interestingly, none of them has been characterized as dominant according to criteria established by Sercarz et al. (Sercarz et al. 1993). The large molecular size of Tg (660 kDa in its dimeric form) and the lack of posttranslational modifications on candidate synthetic peptides have been mentioned as two possible reasons to account for this rather paradoxical finding (Carayanniotis 2003, Carayanniotis 2007).

The large size of the Tg molecule affords it two main functions: 1) Tg acts as a scaffold to allow the formation of T3 and T4 by intramolecular coupling of iodotyrosines, and 2) Tg provides a substrate for selective endoproteolytic cleavages that may facilitate the release of these hormones (Dunn et al. 1991, Gentile et al. 1992, Rousset et al. 1989). The cleavage sites most likely represent conserved, solvent-exposed regions of the primary structure and occur within sequences that disrupt the Cys-rich, tandem repeats of Tg (Gentile, Salvatore 1993). Limited digestion of rabbit Tg by human cathepsins B, D and L has identified major cleavage sites (Dunn et al. 1991) that are near those observed after digestion with different enzymes, supporting the view that the lysosomal degradation of Tg is governed by the intrinsic susceptibility of some of its regions to various proteases (Gentile, Salvatore 1993). With the rationale that the presence of an enzymatic cleavage site near an epitope is a factor enhancing its immunodominance (Sercarz et al. 1993), we

have searched in this report for pathogenic T cell epitopes localized in the proximity of mTg sequences homologous to those encompassing the cathepsin cleavage sites in rabbit Tg. We have looked for A^k-binding motifs within 20-a.a. residue segments adjacent to the homologous murine cathepsin cleavage sites. The immunopathogenicity and immunodominance of 12-mer peptides encompassing these motifs were examined in this study.

3.3. Results

3.3.1. Prediction of H2A^k-binding peptides in mTg.

Because cathepsin L, B, and D cleavage sites in rabbit Tg have been previously described (Dunn et al. 1991), we identified the homologous sites in the mTg sequence. Four cathepsin L cleavage sites in mTg were identified with the P'1 residue at a.a. positions 2388, 2451, 2490 and 2657 (**Table 3.1**). In a similar manner, a.a. 531, 795 and 2486 were defined as P'1 residues for cathepsin B, and a.a. 550, 1833, 2467, and 2642 as P'1 residues for cathepsin D. We then searched for the presence of A^k-binding motifs within 20 a.a. residues from the above sites by scanning the mTg sequence (Kim et al. 1998) with an algorithm described by Altuvia *et al*. (Altuvia et al. 1994). This approach allowed us to delineate five peptides (**Table 3.2**) encompassing such motifs: 2 peptides (a.a. 557-568 and a.a. 1822-1832) were found near 2 putative cathepsin D cleavage sites, 2 peptides (a.a. 2369-2380 and a.a. 2439-2450) near a cathepsin L cleavage site, and 1 peptide (a.a. 771-781) near a cathepsin B cleavage site. The relative position of these mTg peptides vis a vis the location of the putative cathepsin cleavage sites is shown schematically in **Figure 3.1.**

Table 3.1. Possible cathepsin L, B and D cleavage sites in mTg

Endopeptidase	P_4-P_1 sequence[a]	P'_1-P'_{n+1} sequence[b]	P'_1 residue no.[a]
Cathepsin L	QLFR	KALLMGGSALS	2388
	KLLA	VSGPFHYWGPVVDGQ	2451
	LIGG	SQDDGLINRA	2490
	FSRK	A.A.EFATPWP	2657
Cathepsin B	RVVG	NFGFKVNLQENQDAL	531
	REVA	SRNFSLFLQ	795
	KVDL	LIGGSQDDGLINRA	2486
Cathepsin D	FLVS	SLLELPEFLVFLQR	550
	ATEL	FSPVDITQVI	1833
	DGQY	LRELPSRRLKRPL	2467
	FSNF	IRSGNPNYPHEFS	2642

[a] A.a. coordinates are based on the cDNA- derived sequence of mTg by Kim *et al*. (Kim et al. 1998)

[b] Homologous rabbit Tg residues identified by Dunn *et al.* (Dunn et al. 1991) are underlined.

Table 3.2. Amino acid co-ordinates and sequences of mTg peptides used in the study.

Amino acid coordinates[a]	Motif-containing sequence	Peptide denotation	Putative cleavage site P'
557-568	FLVFLQRAVSVP	p557	552 (cathepsin D)
771-781	QNGDGQELTPA	p771	795 (cathepsin B)
1822-1832	SDFPG**DMATE**L	p1822	1833 (cathepsin D)
2369-2380	DVASIHLLISRP	p2369	2388 (cathepsin L)
2439-2450	NILNDAQTKLLA	p2439	2451 (cathepsin L)

[a] A.a. coordinates are extrapolated from the mTg sequence according to Kim *et al*. (Kim et al. 1998) and do not include the leader sequence. Three additional peptides were identified by this methodology but were not used in the study for technical reasons: p1838-1849 had a solubility problem; p2467-2477 carried a proline within a motif sequence; and p2490-2504 was overlapping significantly with the previously identified pathogenic T-cell epitope 2495-2504 (Rao et al. 1994).

[b] The underlined sequences represent A^k-binding motif A: [DEHNQ]-{NQ}-X-[ILTV]-X-{FHWY}-[ACFILMTVWY]; and the sequences in bold represent A^k-binding motif B: [CDEHNQY]-{DE}-X-[ILTV]-[DEHQN] where [ ] represents inclusion and {}represents exclusion of the indicated amino acids at this position; X denotes any amino acid (Altuvia et al. 1994).

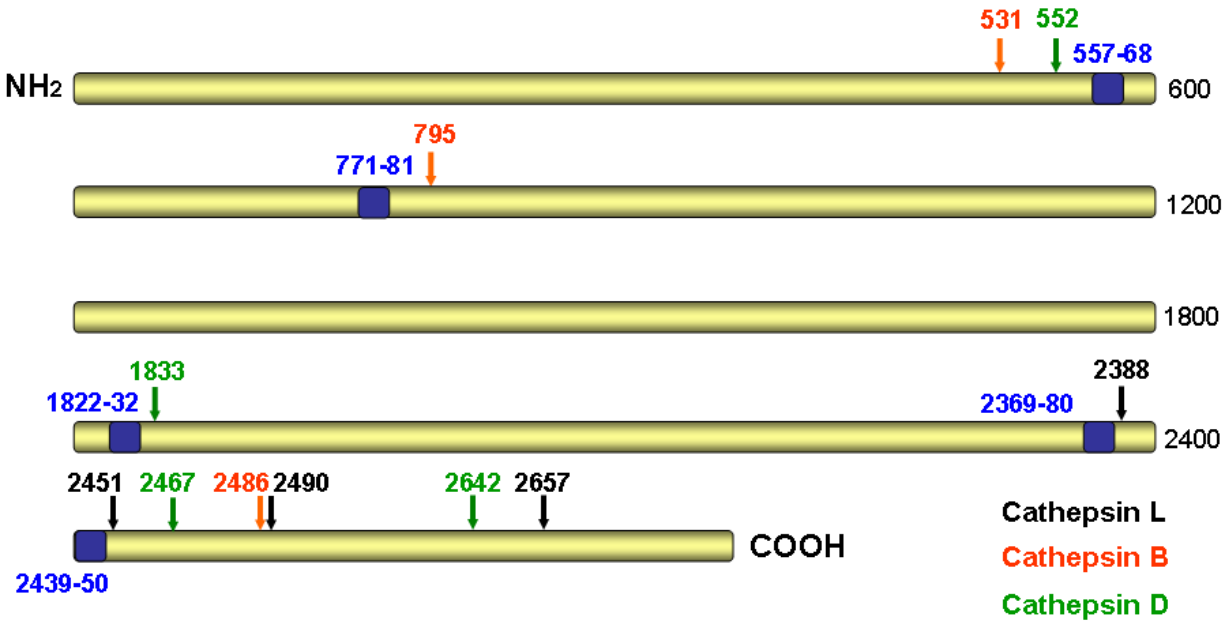

Figure 3.1

Relative position of Tg peptides (shown in blue) used in this study vis a vis the location of putative cathepsin L, B and D cleavage sites (arrows) in mTg based on homology to known cleavage sites in rabbit Tg (Dunn et al. 1991). Peptides were identified by the presence of A^k-binding motifs within 20 a.a. from the cleavage sites, after scanning the mTg sequence with the computerized algorithm described by Altuvia *et al*. (Altuvia et al. 1994).

3.3.2. Assessment of Tg peptide immunogenicity and immunodominance.

To examine the immunogenicity of the Tg peptides, we challenged CBA/J mice s.c. with 100 nmol of each peptide. Inguinal, axillary and branchial LNCs were collected 9 days later and cultured in the presence of the immunizing peptide. Peptides p2369 and p2439, induced significant specific LNC proliferation compared to undetectable responses to control peptide p1579 (**Figure 3.2A**), indicating that these Tg peptides contain epitopes that elicit T cell responses in CBA/J mice. To examine the immunodominance of T cell determinants contained in these peptides, we challenged CBA/J mice with 100 μg of Tg in CFA and 9 days later, LNCs were collected and cultured in the presence of Tg or free p2369 or p2439. As shown in **Figure 3.2B**, LNCs from Tg-primed mice responded to Tg but failed to respond to equimolar (0.2 - 0.9 μM range) concentrations of free peptide, with responses remaining undetectable at peptide concentrations as high as 75 μM. Conversely, LNCs from peptide-primed mice responded *in vitro* to the immunizing peptide but did not respond to Tg (**Figure 3.2C**), indicating that p2369 and p2439 do not contain dominant T cell epitopes. The cytokine production profile of peptide-reactive LNCs from p2369- and p2439-primed CBA/J mice showed secretion of IL-2 and IFN-γ (**Figure 3.3**), but no IL-4 detection (data not shown), suggesting the activation of Th1 cells. No cytokine secretion was detected upon culturing the above peptide-primed LNCs with the unrelated p1579 peptide control (not shown).

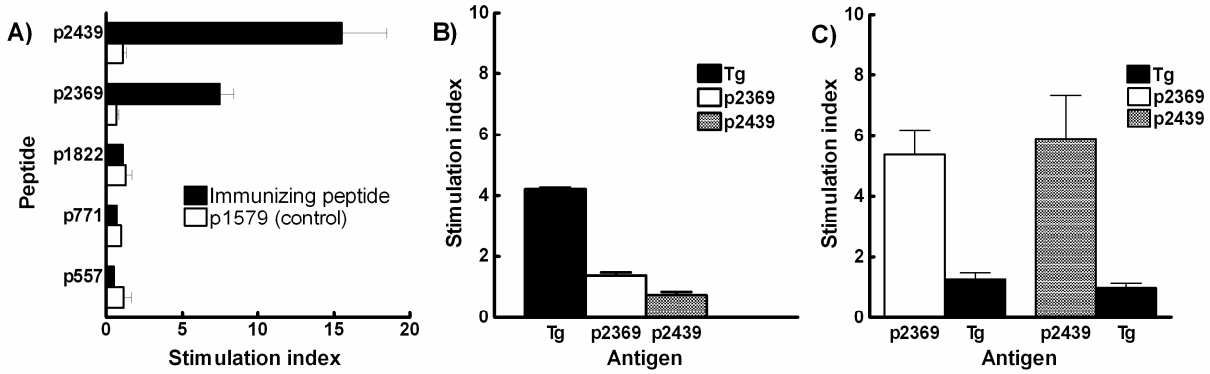

Figure 3.2

Secondary proliferative responses *in vitro* of LNC from: A) peptide-primed CBA/J mice in the presence of 5 μM of the immunizing peptide or p1579 control. Data were obtained from full peptide titration curves (0.5 – 25 μM) and are representative of four independent experiments; B) Tg-primed CBA/J mice against Tg or equimolar concentration (1 μM) of the peptide antigen shown; C) Peptide-primed CBA/J mice in the presence of 1 μM of the immunizing peptide or Tg. Data show the mean S.I. of triplicate wells ± S.D.

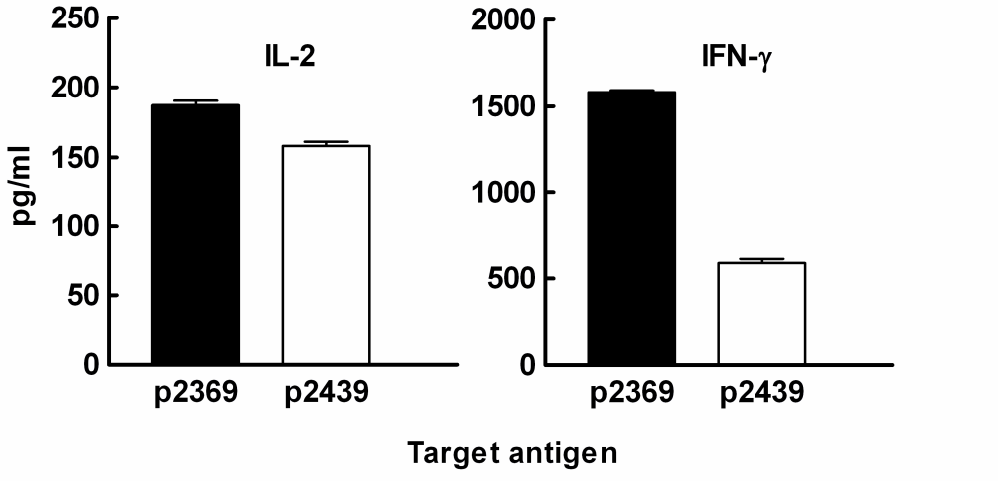

Figure 3.3

Cytokine profile of Tg peptide-reactive LNC as determined by sandwich ELISA after 48 hour culture in the presence of 25 μM of the respective peptide shown. IL-2 (A) and IFN-γ (B) concentrations were extrapolated from standard curves. Cytokine production in response to p1579 peptide control was at background levels (17-22 pg/ml for IL-2 and < 40 pg/ml for IFN-γ). Results represent the mean values of triplicate wells ± S.D.

3.3.3. Assessment of Tg peptide pathogenicity.

CBA/J mice were subsequently challenged with p2369 (n=8) or p2439 (n=7) in CFA and 3 weeks later were boosted with the same peptide in IFA. Two weeks after the last immunization, thyroid glands were histologically examined for EAT development. Both peptides were pathogenic inducing EAT with low incidence (4/8 mice with EAT after challenge with p2369 and 2/7 mice with EAT after challenge with p2439) and severity (mean I.I.) ranging from 0.4-0.6; **Table 3.3**). Thyroiditogenicity was confirmed when adoptive transfer of p2369-specific effector cells into naïve hosts induced EAT in 3/5 mice with mean I.I. = 0.6, and that of p2349-specific cells elicited EAT in 6/6 mice with mean I.I. = 1.5 (**Table 3.3**). In contrast, neither peptide induced peptide-specific IgG responses (data not shown).

Table 3.3. EAT induction by Tg peptides in CBA/J mice.

Direct induction of EAT[a]						Adoptive transfer of EAT[b]					
I.I.						I.I.					
Peptide	0	1	2	No of mice with EAT	Mean I.I.	Peptide	0	1	2	No of mice with EAT	Mean I.I.
p2369	4	3	1	4/8	0.6	p2369	2	3		3/5	0.6
p2439	5	1	1	2/7	0.4	p2439	0	3	3	6/6	1.5

[a] CBA/J mice were challenged s.c. with 100 nmol of the indicated peptide and 3 weeks later boosted with 50 nmol of the peptide in IFA. Thyroids were removed 2 weeks after the last immunization as described in Materials and Methods.

[b] Twelve CBA/J mice were challenged s.c. with 100 nmol of the indicated peptide. Nine days later LNC were collected and cultured *in vitro* with 25 μM peptide for 72 hours. Next, cells were transferred i.p. into syngeneic recipients (6 mice per group) and thyroids were collected 14 days later.

3.4. Discussion

In this study, we have used sequence homology comparisons to correspond known cathepsin L, B and D cleavage sites in rabbit Tg (Dunn et al. 1991) with their counterparts in the mTg sequence, and we examined whether putative A^k-binding peptides located near these cleavage sites would have immunodominant and immunopathogenic properties in CBA/J mice. Our data describe two new thyroiditogenic Tg epitopes, p2369-2380 and p2439-2450, located near the cathepsin L cleavage sites 2388 and 2451, respectively. Interestingly, these epitopes are clustered close to known immunopathogenic Tg epitopes p2494-510 (Chronopoulou, Carayanniotis 1992), p2495-503 and p2498-506 (Rao et al. 1994) in mTg and p2340-59 in hTg (Karras et al. 2003, Karras et al. 2005) at the C-terminal region of the large Tg molecule. Recent work by Tomer's group (Jacobson et al. 2009) examining hTg digests by individual human cathepsins L, B and D identified cleavage sites near the ones described by earlier work of Dunn et al. with rabbit Tg in 7 out of 11 cases shown in Table 1. Also, it is interesting that work with mTg digests by Dedieu et al. (Dedieu et al. 2011) has confirmed that the a.a. sites 2368-71 (ADVA motif) and a.a. 2410-11 (a.a. co-ordinates do not include the 20 a.a. leader sequence), located at the start or near our identified peptides, are very sensitive to proteolytic attack. That study revealed that six out of the 11 cleavage sites shown in **Table 1** were near to or overlapping with the N-termini of peptide fragments solubilized from mouse thyroid lobe extracts. These, as well as earlier (Gentile et al. 1992, Gentile, Salvatore 1993) findings give support to the notion that the Tg substrate(s) recognized by proteolytic enzymes within thyrocytes may be conserved among species.

The rationale that immunodominant epitopes are likely to be found near proteolytic sites was based on earlier work with antigens such as hen egg lysozyme (Schneider et al. 2000) and myelin basic protein (Manoury et al. 2002, Anderton et al. 2002). However, the immunodominant peptide(s) in mTg remain elusive as neither of p2369/p2439 appeared to be generated by the processing of intact mTg *in vivo* or *in vitro*. The paradoxical finding that no one of the 27 (including this study) known pathogenic Tg peptides exhibits clear immunodominant behavior stems from using an arbitrary definition since, by convention, we expect such a peptide to be generated following processing of intact Ag in peripheral APC. Parameters involved in this process can be categorized at two levels: 1) The level of the substrate; and 2) the level of the APC involved.

At the level of the substrate, it has been suggested that the large size of Tg (consisting of two identical subunits of 330 kDa) may prohibit the quantitative yield of any given average (2 kDa) candidate peptide following Tg processing in peripheral APC (Carayanniotis 2003, Carayanniotis 2007). Also, requirements for post-translational modifications of epitopes such as glycosylation, phosphorylation or iodination are not usually been taken into account by the screening assays (Carayanniotis 2007). A third view argues that the relevant peptidic substrate for APC (intrathyroidal or peripheral) is not intact Tg per se, but rather Tg fragments released from thyrocytes. In this case, the epitopes under consideration could be dominantly expressed due to an alteration of the

immunodominance hierarchy in the shorter Tg fragments. This is a plausible explanation, given that only very small amounts of intact Tg are secreted from the basolateral membrane of thyrocytes during transepithelial transport (transcytosis) – a process mediated by megalin which protects Tg from lysosomal degradation (Marino et al. 2001). This process competes with the main proteolytic pathway of Tg in lysosomes which is orchestrated to maximize hormone recovery under a regimen of limited proteolysis. In this regard, working with SDS-PAGE bands of mouse thyroid extracts comprising proteins with molecular masses lower than 250 kDa, Dedieu *et al*. identified 174 different peptides corresponding to Tg fragments (Dedieu et al. 2011). Thus, although the relative amounts of intact Tg vs Tg fragments in the secreted peptidic repertoire are not known, it is conceivable that Tg fragments may be the main substrate of relevance in the aetiopathogenesis of disease. Unfortunately, a "model Tg peptidic repertoire" that could be used in studies of immunodominance of given Tg epitopes remains obscure. Tg degradation is a complex process, starting with the solubilization of stored Tg from covalently cross-linked globules by cathepsins B and L, limited digestion by cathepsins K and L for extracellular T4 liberation (in the lumen) and final re-entry into endo-lysosomes for complete proteolysis by several lysosomal enzymes (Friedrichs et al. 2003). In addition, several proteases may act in concert, and their activities may be influenced by their subcellular location (Jordans et al. 2009) or by protease-resistant segments (type 1 repeats) of Tg itself (Molina et al. 1996, Mihelic, Turk 2007, Gentile et al. 2004).

At the level of APC, DC - intrathyroidal or within thyroid-draining LN - are likely to be the primary cells involved in the MHC-class II presentation of Tg peptides derived from a Tg fragment substrate. Proteolytic action within BM-derived APC would be distinct from that occurring within thyrocytes as cathepsin L and S mediate the late stages of invariant chain degradation (Nakagawa et al. 1998, Nakagawa et al. 1999, Riese et al. 1998, Shi et al. 1999). A developing immune response would further complicate the regulation of this process, since IFN–γ is known to enhance the role of cathepsin S and inhibit cathepsin L activity in APC (Beers et al. 2003, Guncar et al. 1999). As a consequence of an inflammatory response, thyroid epithelial cells could also become APC – once they acquire antigen-presenting capability through IFN-γ-induced MHC class II upregulation (Londei et al. 1984, Bottazzo et al. 1983) – and new sets of Tg peptides, endogenously processed in thyrocytes may be recognized by autoreactive T cells. In this regard, work with human T cell clones, derived from thyroid infiltrates and specific for TPO epitopes, has supported the notion that thyrocytes as APC present a different TPO peptide repertoire from that obtained by the processing of the exogenous antigen in blood mononuclear cells (Quaratino et al. 1996).

In this and earlier work, the recognition of a plethora of Tg peptides by pre-activated specific effector T cells that home to the thyroid, after adoptive transfer in the mouse EAT model, formulates two important corollaries: First, it clearly argues that these epitopes must be intrathyroidally expressed under steady-state conditions, perhaps on resident DC (Voorby et al. 1990). This view is supported by data published by Unanue's

group in autoimmune diabetes which show that DC in islets of Langerhans constitutively present β cell-derived peptides bound to class II MHC molecules (Calderon et al. 2008, Calderon, Unanue 2012). Constitutive presentation of a "constellation" of Tg peptides to the immune system may contribute to the maintenance of peripheral tolerance which could be abrogated by conditions that favor the generation of effector cells in the periphery. Second, for unknown reasons, the proximity of peptides such as 2369-2380 and 2439-2450 to proteolytic cleavage sites may facilitate their processing and presentation on MHC class II antigens without necessarily promoting their destruction by exopeptidase activity.

In view of the above, it is remarkable that traditional methods of epitope mapping applied to the large mTg molecule in the field of mouse EAT have identified peptides that may have biological relevance to the pathogenesis of human autoimmune thyroid disease. For example, a large homologous segment of the mTg pathogenic peptide p2495-2511, initially identified by an algorithm searching for the presence of mouse MHC-binding motifs (Chronopoulou, Carayanniotis 1992) was eluted from DR molecules in thyroid samples from patients with Graves' disease (Muixi et al. 2008a), and overlapped significantly with a hTg peptide obtained after digestion with human cathepsin B (Jacobson et al. 2009). Also, diverse screening methodologies have independently resulted in the delineation of identical pathogenic peptides. For example, the pathogenic mTg peptide 179-194 that was initially identified for encompassing an A^k-binding motif flanked by a Tyr residue (Li et al. 2007), was subsequently found to be a major

thyroiditogenic peptide in the A-E+ transgenic model of EAT (Brown et al. 2008a). These examples strongly suggest that, currently, there is no single best methodology for the continuing discovery of pathogenic Tg peptides and that any clinical implications stemming from the EAT data will slowly emerge as human studies progress in the future.

CHAPTER 4

Apoptosis of NOD.H2^{h4} thyrocytes by low concentrations of iodide is associated with impaired control of oxidative stress

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4.1. Abstract

Enhanced iodide intake in NOD.H2^{h4} mice accelerates the incidence and severity of SAT (ISAT) via an unknown mechanism. A plausible hypothesis is that iodide-induced apoptosis of thyrocytes can create imbalances in antigenic load and/or disruption of immunoregulatory mechanisms that facilitate activation of autoreactive T cells in CLN draining the thyroid. We have examined whether NOD.H2^{h4} thyrocytes, exposed to low NaI concentrations *in vitro*, are more susceptible to apoptosis compared to thyrocytes from CBA/J mice which are resistant to ISAT. We have also looked, at the transcriptional level, for differential activation of genes involved in apoptosis or oxidative stress pathways that may account for potential differences in iodide-mediated apoptosis between NOD.H2^{h4} and CBA/J thyrocytes. We report that NOD.H2^{h4} thyrocytes, cultured for 24 hr at very low (4-8 μM) concentrations of NaI, exhibit high levels (40-55%) of apoptosis, as assessed microscopically following staining with fluorescent caspase inhibitors. Similar treatment of thyrocytes from CBA/J mice which are resistant to ISAT yielded significantly lower (10-20%) apoptotic rates. Expression analysis by real-time PCR using arrays of apoptosis- and oxidative stress-related genes showed that NaI intake upregulates the expression of 22 genes involved in ROS metabolism and/or anti-oxidant function in CBA/J thyrocytes whereas only two of these genes were upregulated in NOD.H2^{h4} thyrocytes. Among the set of overexpressed genes were those encoding TPO (*Tpo*) (5.77 fold), glutathione peroxidases (*Gpx2, Gpx4, Gpx7*), (2.03 – 3.14 fold), peroxiredoxins (*Prdx1, Prdx2, Prdx5*) (2.27 - 2.97 fold), superoxide dismutase 1 (*Sod1*) (3.57 fold), thioredoxin 1 (*Txn1*) (2.13 fold) and the uncoupling proteins 2 and 3 (*Ucp2,*

Ucp3) (2.01-2.15 fold). The results demonstrate that an impaired control of oxidative stress mechanisms is associated with the observed high susceptibility of NOD.H2^{h4} thyrocytes to NaI-mediated apoptosis and suggest a contributing factor for the development of ISAT in this strain.

4.2. Introduction

The NOD.H2^{h4} mouse strain has been well established as an animal model for the study of SAT. This strain was originally made in 1993 to investigate potential effects of H-2 region genes on diabetes but it was serendipitously found to develop only insulitis and not diabetes (Podolin et al. 1993) and a much higher incidence (50%) of SAT vs. 5% in NOD mice (Wicker et al. 1995, Burek, Rose 2008). The high incidence of SAT in this strain, carrying the K^k, A^k, E^0, D^b haplotype, has been attributed in part to the combined presence of the A^k molecule which is permissive for thyroiditis development (Kong et al. 1997) and NOD non-MHC genes that are known to predispose to autoimmunity (Wicker 1997). SAT appears after 15 weeks of age and its incidence and severity increase progressively with time. However, when 6-8 wk old NOD.H2^{h4} mice are placed on drinking water containing 0.05% NaI, SAT becomes detectable as early as 2 weeks after the initiation of the dietary regimen and plateaus within 4-8 weeks. The discovery of ISAT in NOD.H2^{h4} mice complements similar earlier observations of thyroiditis induction by iodide in NOD mice (Hutchings et al. 1999, Vecchiatti et al. 2013), BB/W rats (Allen et al. 1986) and OS chickens (Bagchi et al. 1985). These animal models strongly suggest the interplay between dietary (environmental) and genetic factors in the activation of an autoimmune response and are likely to involve processes similar to those that precipitate development of autoimmune thyroiditis and hypothyroidism in humans following excessive iodine intake (Konno et al. 1994, Markou et al. 2001, Bournaud, Orgiazzi 2003).

Intrathyroidal infiltration by mononuclear cells and production of Tg-specific IgG Abs have been well established as the cardinal symptoms of ISAT in NOD.H2^{h4} mice (Hutchings et al. 1999, Braley-Mullen et al. 1999, Rasooly et al. 1996) but the early events whereby iodide triggers the autoimmune cascade remain unknown. Thyrocytes actively take up iodide through the function of the NaI symporter (Provenzano et al. 2009) and is well known that their exposure to high concentrations of iodide can induce their apoptosis or necrosis *in vivo* (Bagchi et al. 1985, Teng et al. 2009, Li, Boyages 1994, Chen et al. 2011), or *in vitro* (Many et al. 1992, Lehmann et al. 2006, Yao et al. 2012, Vitale et al. 2000). A plausible hypothesis is that iodide-induced apoptosis of thyrocytes can create imbalances in antigenic load and/or disruption of immunoregulatory mechanisms that facilitate activation of autoreactive T cells in CLN draining the thyroid. In this report, we have examined whether NOD.H2^{h4} thyrocytes, exposed to low NaI concentrations *in vitro*, are more susceptible to apoptosis compared to thyrocytes from CBA/J mice which are resistant to ISAT (Li, Carayanniotis 2007). We have also looked, at the transcriptional level, for differential activation of genes involved in apoptosis or oxidative stress pathways that may account for potential differences in iodide-mediated apoptosis between NOD.H2^{h4} and CBA/J thyrocytes.

4.3. Results

4.3.1. Establishment of primary thyroid epithelial cell culture and assessment of apoptosis

Single cell suspensions from NOD.H2^{h4} thyroid glands were prepared and cultured for 10 days in complete Ham F-12 medium promoting epithelial cell growth, as described in Materials and Methods. Using a two-step immunofluorescence assay with FITC-labeled goat anti-mouse IgG, it was shown that over 98% of the grown cells were thyrocytes since their cytoplasm was positively stained by thyroglobulin-specific IgG antibodies (**Figure 4.1A**) but remained unstained by normal serum IgG (**Figure 4.1B**). In preliminary experiments, apoptosis of thyrocytes, cultured in the presence of various NaI concentrations, was assessed with the FAM-FLICA^{TM} *in vitro* Poly Caspase kit which uses a reagent that penetrates the cell membrane and binds irreversibly to activated caspases. With this method, we could readily enumerate healthy (**Figure 4.1C**) vs. early or late apoptotic thyrocytes (**Figure 4.1D, E**). Necrotic thyrocytes were identified by staining with propidium iodide (**Figure 4.1F**).

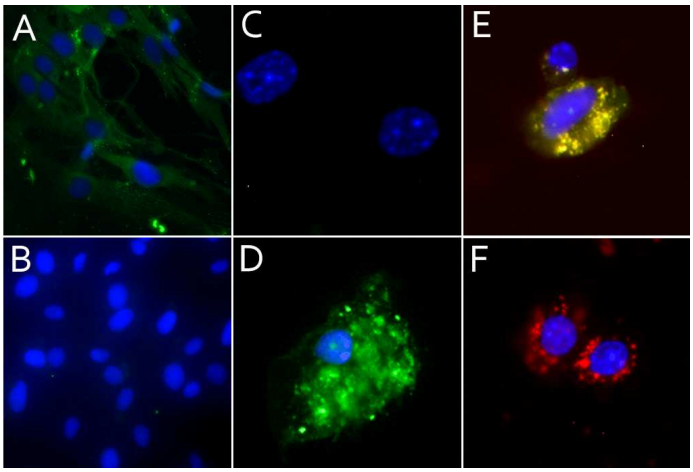

Figure 4.1

Establishment of primary mouse thyrocyte cultures and assessment of NaI-induced apoptosis. NOD.H2^{h4} thyrocytes were expanded in culture for 10 days and their purity was assessed by a two-step immunofluorescence assay using FITC-labeled goat anti-mouse IgG, as described in *Materials and Methods*. (A) Thyrocytes incubated with NOD.H2^{h4} serum containing high titers of Tg-specific IgG; (B) thyrocytes incubated with control serum from healthy CBA/J mice. Appearance of NOD.H2^{h4} thyrocytes following exposure to apoptotic/necrotic stimuli. (C) Healthy thyrocytes (blue staining of nucleus with Hoechst 33342)**;** (D) Early apoptotic thyrocyte cultured in 10^{-5} M NaI (green fluorescent staining of cytoplasm with FAM-FLICA poly caspase reagent); (E) late apoptotic thyrocyte cultured in 10^{-5} M NaI (cytoplasmic stain with FAM-FLICA and propidium iodide); (F) necrotic thyrocyte following exposure to 90% ethanol for 30 sec (cytoplasmic stain with propidium iodide).

4.3.2. Low concentrations of iodide induce differential apoptosis in NOD.H2^{h4} vs CBA/J thyrocytes.

The effect of iodide on NOD.H2^{h4} thyrocyte viability *in vitro* was initially tested within a large range of final NaI concentrations (10^{-5} – 10^{-3} M). NaI was added to 9-day cell cultures for 24 hours and subsequently apoptosis was microscopically assessed with the FAM-FLICA assay by counting 300 cells from cultures at each NaI concentration in five independent experiments (**Figure 4.2**). It was found that the background apoptosis of thyrocytes grown in the absence of NaI was relatively high (27.12 ± 3.1) (percentage mean +/- SEM) and that exposure to 10^{-5} M NaI significantly increased the percentage of total apoptotic cells to 58.34 ± 6.3, (p= 0.021). This level of apoptosis remained relatively constant over the 10^{-5} -10^{-3} M NaI range and was comparable to that observed following a 12 hour treatment of thyrocytes with 6 μM camptothecin (59 ± 3.6). The number of necrotic cells in all cultures was negligible. On the basis of these initial data we selected the 1 x 10^{-6} – 1.6 x 10^{-5} M NaI range to compare NOD.H2^{h4} vs CBA/J thyrocytes in terms of their susceptibility to iodide-induced apoptosis since CBA/J mice do not develop ISAT following high NaI intake. As shown in **Figure 4.3**, in the absence of NaI, NOD.H2^{h4} thyrocytes showed a background apoptotic rate of 21.59 ± 4.00 which was not significantly higher than that observed in the corresponding CBA/J cell culture, (11.5 ± 0.5, p = 0.128). However, increasing NaI concentrations induced higher apoptotic rates in NOD.H2^{h4} thyrocytes, peaking at 54.45 ± 5.45 at 8 x 10^{-6} M NaI. In contrast, over the same molar range of NaI, CBA/J thyrocytes exhibited total apoptotic cell levels which were not significantly higher than those of the control (no NaI) cultures. The percentage

of necrotic cells in all cultures was negligible (not shown) and never exceeded 3.4%. Possible osmotic effects were excluded because after exposure to equimolar NaCl concentrations, apoptotic cell rates did not rise above background values (not shown). These data demonstrated that, at low NaI concentrations *in vitro*, NOD.H2^{h4} thyrocytes are more sensitive to iodide-mediated apoptosis than CBA/J thyrocytes which appeared to be unaffected by the NaI treatment.

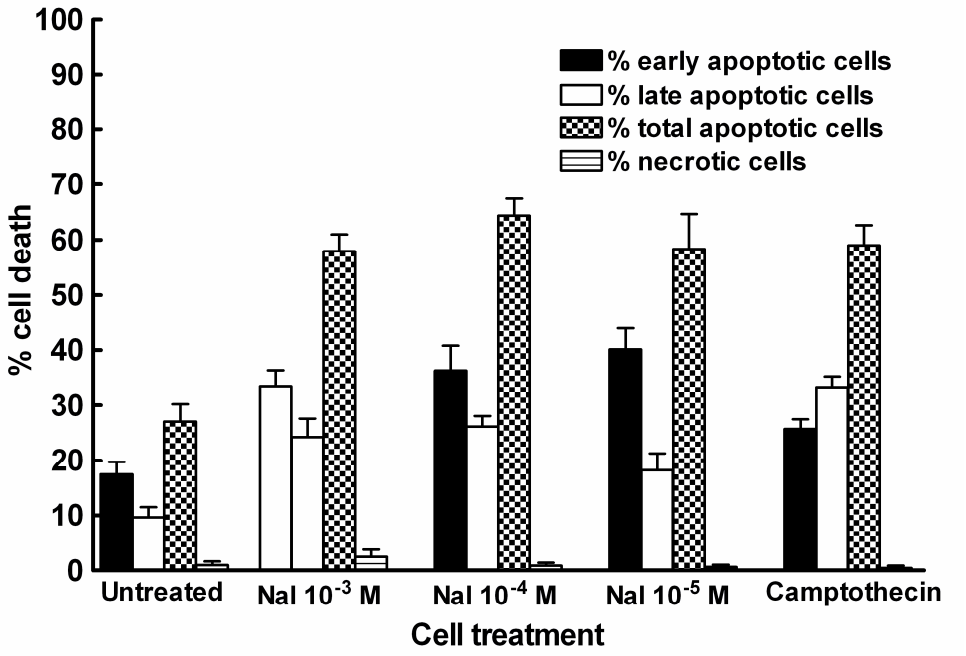

Figure 4.2

Death of cultured NOD.H2^{h4} thyrocytes following 24 hour treatment with the indicated concentrations of NaI or camptothecin control (12 hr, 6 μM). Apoptosis or necrosis was microscopically assessed by examination of at least 300 cells per group after staining as described. The data indicate mean ± SEM of 5 experiments. The statistical significance between the untreated controls and the NaI-treated groups assessed by a paired t-test was as follows: NaI 10^{-3} M, p=0.019; NaI 10^{-4} M, p=0.001; NaI 10^{-5} M, p=0.021.

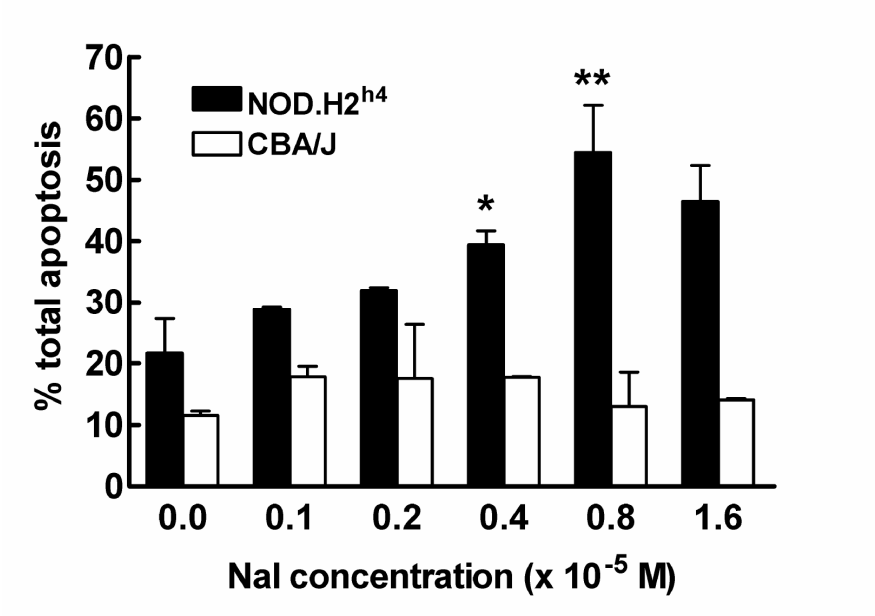

Figure 4.3

NOD.H2^{h4} thyrocytes are highly sensitive to iodide-induced apoptosis. Day 9 NOD.H2^{h4} and CBA/J thyrocyte cultures were exposed to the indicated final NaI concentrations for 24 hours. Total (early and late) apoptotic cells were microscopically viewed and counted as described in Figure 1, using the FAM-FLICA assay. Three hundred cells were examined per group and data represent percent apoptosis mean ± SEM of two independent experiments. The percentage of necrotic cells in all cultures never exceeded 3.4%. Statistical significance was determined by a two-tailed unpaired t-test. * $p = 0.051$; ** $p = 0.025$.

4.3.3. Analysis using apoptosis PCR arrays

To further investigate the iodide-mediated apoptosis pathway, we looked at the expression of 84 apoptosis-related genes (**Appendix 1**) by real time PCR using a mouse Apoptosis PCR array. We first examined whether the number or the relative constitutive expression of pro-apoptotic genes was higher in NOD.H2^{h4} vs. CBA/J thyrocytes. Within a set of 35 apoptosis-promoting genes, significant expression differences were observed at eight genes, with two genes (*Tnf* and *TnfSF10*) showing higher expression and six genes (*Traf 2, Trp73, Card 10, Casp 9, Dffb,* and *Casp6*) showing lower expression in NOD.H2^{h4} vs. CBA/J/ thyrocytes (**Table 4.1**). Conversely, within a set of 26 apoptosis-inhibiting genes, significant expression differences were observed at four genes, with one gene (*Naip2*) showing higher expression (3.29-fold) and three genes (*Nme5, Cidea, BCl2l10*) showing lower expression in NOD.H2^{h4} vs. CBA/J/ thyrocytes (**Table 4.1**). These data did not support the view that NOD.H2^{h4} thyrocytes intrinsically express a clear apoptosis-promoting profile at the gene transcription level, as compared to CBA/J thyrocytes.

Next, we examined the effects of NaI on gene transcription rates in thyrocytes from each strain. After nine days of culture, cells were treated with 4 x 10^{-6} M NaI and RNA from these - and from untreated thyrocyte controls - was extracted 24 hours later. Surprisingly, in NOD.H2^{h4} thyrocytes only the pro-apoptotic gene *bok* (BCL-2 related ovarian killer protein), showed significant alteration in its expression as it was completely suppressed (> - 500-fold) (**Appendix 1**). Interestingly, expression of several genes that belong in the

Bcl-2 family, and various caspases and their activators, was not significantly affected by the iodide treatment. In NaI-treated CBA/J thyrocytes, there was mild downregulation of five pro-apoptotic genes (*Abl1, Dffb, RipK1, Casp9, Card 10*) and three anti-apoptotic genes (*Bcl2l0, Atf5, Il10*) vs. the untreated controls (**Appendix 1).** Only 1 pro-apoptotic gene (*Tnfsf10*) was upregulated (2.36-fold). Taken together, these data suggested that the apoptosis-enhancing effect of NaI, differentially observed in NOD.H2^{h4} vs. CBA/J thyrocytes in **Figure 3**, could not be clearly attributed to factors operating at the transcriptional level of the genes tested.

Table 4.1. Differential expression of apoptosis-related genes in NOD.H2^{h4} and CBA/J thyrocytes

RefSeq	Symbol	Description	Fold change (NOD.H2^{h4}/CBA/J) [a]
		Apoptosis promoting genes	
NM_013693	Tnf	Tumor necrosis factor	3.04
NM_009425	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	2.95
NM_009422	Traf2	Tnf receptor-associated factor 2	-2.01
NM_130859	Card10	Caspase recruitment domain family, member 10	-2.89
NM_015733	Casp9	Caspase 9	-3.03
NM_007859	Dffb	DNA fragmentation factor, beta subunit	-4.01
NM_009811	Casp6	Caspase 6	-23.85
		Apoptosis inhibiting genes	
NM_010872	Naip2	NLR family, apoptosis inhibitory protein 2	3.29
NM_080637	Nme5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	-2.00
NM_007702	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	-2.57
NM_013479	Bcl2l10	Bcl-2-like 10	-2.66

[a] A fold change $\geq$ 2.00 (upregulation) and $\leq$ -2.00 (downregulation) was considered significant.

4.3.4. Analysis using oxidative stress PCR arrays

Enhanced iodide-induced apoptosis in NOD.H2^{h4} thyrocytes could be triggered by high levels of ROS that has been reported to be detected in thyrocytes of this strain in the absence of NaI (Sharma et al. 2008). To examine whether NaI-treated or control NOD.H2^{h4} thyrocytes have a defect in the expression of genes participating in ROS metabolism or anti-oxidant defense mechanisms leading to overproduction of ROS, we performed analysis using an oxidative stress PCR array. The constitutive expression levels of 84 oxidative stress-related genes, (**Appendix 2),** was initially compared between control thyrocytes from the two strains. Significant expression differences were observed at only four genes involved in ROS metabolism, with three genes (*Ncf1, Ncf2* and *Ccl5*) showing higher and one gene (*Duox1*) showing lower expression in the NOD.H2^{h4} group (**Table 4.2**). On the other hand, NOD.H2^{h4} thyrocytes significantly overexpressed three genes (*Gpx2, Cygb, and Mpo*) and underexpressed one gene (*Fancc*) associated with anti-oxidant function (**Table 4.2**).

When the effects of NaI on oxidative stress-related gene transcription rates were examined in thyrocytes from each strain, the results were striking. In CBA/J thyrocytes, NaI upregulated the expression of 22 genes involved in anti-oxidant defense mechanisms and ROS metabolism whereas the same treatment increased expression of only two of these genes (*Gpx2*, glutathionine peroxidase 2, and *Nqo1*, NADPH dehydrogenase, quinone 1) in NOD.H2^{h4} thyrocytes (**Figure 4.4**). This gene set comprised several

peroxidases, with the main effect observed with *Tpo*, (5.77-fold change). In contrast, NaI mildly upregulated the expression of only one gene (*Noxo1*, NADPH oxidase organizer 1) (2.09-fold change) and downregulated the expression of two genes *Cygb*, (cytoglobin) (-2.17-fold) and *Krt1*, (keratin 1), (-3.7-fold) in NOD.H2^{h4} cells. These results strongly suggested that an impaired capacity to mobilize molecules with anti-oxidant function might explain in part the observed susceptibility of NOD.H2^{h4} thyrocytes to apoptosis induced by low concentrations of NaI.

Table 4.2. Differential expression levels of oxidative stress- related genes in NOD.H2^{h4} and CBA/J thyrocytes

RefSeq	Symbol	Description	Fold change (NOD.H2^{h4} / CBA/J)[a]
Genes encoding proteins involved in ROS metAbolism			
NM_010876	Ncf1	Neutrophil cytosolic factor 1	4.52
NM_010877	Ncf2	Neutrophil cytosolic factor 2	2.89
NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	2.40
NM_001099297	Duox1	Dual oxidase 1	-2.74
Genes encoding proteins with anti-oxidant function			
NM_030677	Gpx2	Glutathione peroxidase 2	2.88
NM_030206	Cygb	Cytoglobin	2.51
NM_010824	Mpo	Myeloperoxidase	2.00
NM_007985	Fancc	Fanconi anemia, complementation group C	-4.78

[a] A fold change ≥2.00 (upregulation) and ≤ -2.00 (downregulation) was considered significant.

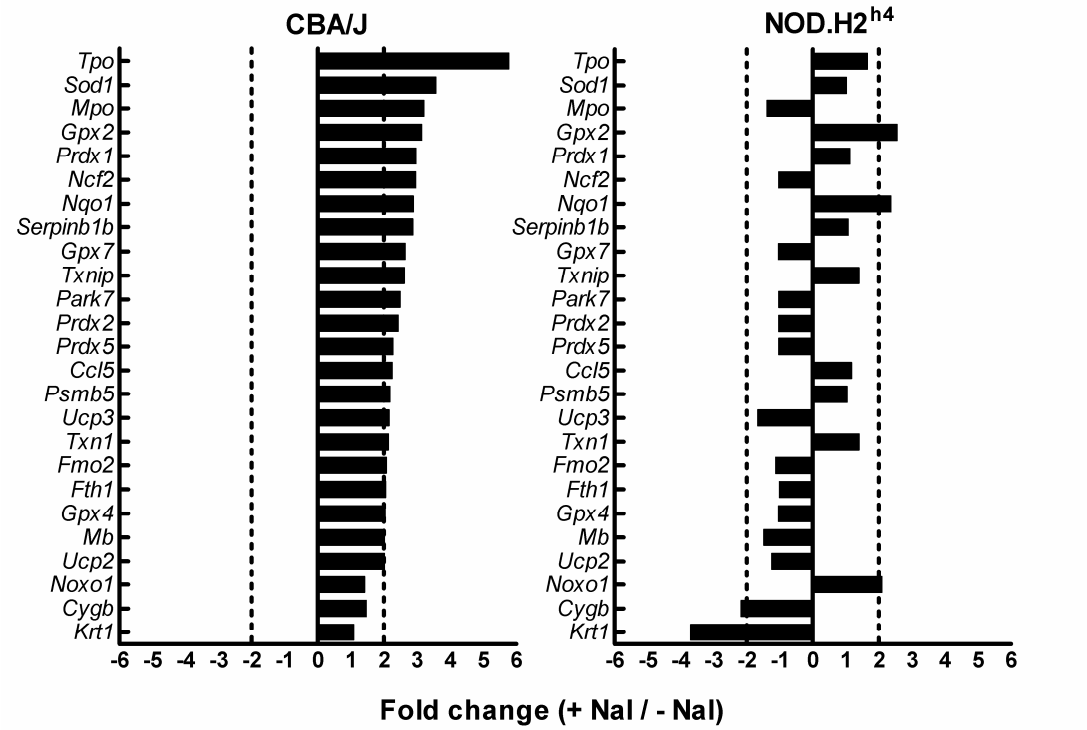

Figure 4.4

Differential expression of oxidative stress-related genes in iodide-treated NOD.H2^{h4} and CBA/J thyrocytes. Thyroid cell suspensions from 80 NOD.H2^{h4} and 80 CBA/J thyroid glands were cultured in vitro for 9 days. On day 9, 4 x 10^{-6} M NaI was added to wells and RNA was isolated 24 hours later. cDNA was used in an Oxidative Stress PCR array and analyzed by real time PCR. Fold change ≥ 2.00 or ≤ -2.00 was considered significant

4.4. Discussion

The goal of this study was to examine whether NOD.H2^{h4} thyrocytes exhibit high sensitivity to NaI-induced apoptosis with the view that this might provide an early trigger in ISAT development in this mouse strain. Work with thyrocytes from euthyroid mice and relatively low doses of NaI could model *in vivo* processes of protracted increased dietary iodine intake in healthy people and in this regard, this approach differs from numerous previous studies which have described iodide-induced cell death in thyrocytes from goitrous animals (Ruwhof, Drexhage 2001). Our data showed that NOD.H2^{h4} thyrocytes, cultured for 24 hours in the presence of 4-8 x 10^{-6} M NaI, underwent apoptosis at very high rates (40 – 55 %) whereas similar treatment of thyrocytes from CBA/J mice – which are resistant to ISAT (Li, Carayanniotis 2007) – did not raise apoptosis above background levels. To our knowledge, such high levels of apoptosis induced by very low concentrations of iodide *in vitro* have not been previously reported. Previous studies with porcine (Langer et al. 2003) or human (Lehmann et al. 2006) thyroid follicles have shown pro-apoptotic effects of iodide in the 2-5 x10^{-6} M range but the observed apoptotic rates were low (4-5%). Usually, very high levels (10^{-4} - 10^{-2} M) of iodide have been used to induce significant cell death in thyroid cell lines such as FRTL-5 cells (Yao et al. 2012, Golstein, Dumont 1996), TAD-2 cells (Vitale et al. 2000) or human thyroid follicles (Many et al. 1992). To a certain extent, variances in apoptotic rates reported by various studies may be due to the use of different assays of apoptosis measurement or even the animal species examined (Golstein, Dumont 1996).

In this study, the enhanced NaI-induced cell death of NOD.H2^{h4} thyrocytes cannot be attributed to necrosis since the levels of necrotic cells in all groups did not exceed 3.4%. The irreversible binding of the fluorescent inhibitor FAM-VAD-FMK to activated caspases 1,3,4,5,6,7,8 and 9 precludes speculation about the possible apoptotic pathways involved, e.g. the participation of inflammasome via activation of caspase-1 or the apoptosome followed by activation of caspase-9 (Latz et al. 2013). Given the extent of apoptosis observed microscopically, it was surprising that the NaI treatment was found not to up- or down-regulate any genes in the apoptotic gene array involved in the intrinsic and extrinsic apoptotic pathways as well as in caspase and DNA damage response cascades, strongly suggesting that this process is independent of regulation at the transcription level and it is executed by proteins already present in the cell. This finding is in agreement with data from a previous study showing that increasing concentrations of cycloheximide, a protein synthesis inhibitor, did not block the apoptosis of the iodide-treated thyroid cell line, TAD-2 (Vitale et al. 2000). Expression of p53, Bcl-2, Bax and Bcl-XL genes has also been reported to remain unchanged at the protein level after 12, 24 and 48 hours of KI treatment of TAD-2 cells (Vitale et al. 2000) while gene array analysis in NIS/TPO modified lung cancer cells has shown that iodide-induced apoptosis did not alter significantly the expression levels of all apoptosis-related genes except *p21* and *survivin* (Zhang et al. 2003). In our study, the apoptotic gene array data derived from NOD.H2^{h4} and CBA/J thyrocytes at the basal (non NaI-treated) state (**Table 4.1**) did not provide clear evidence that NOD.H2^{h4} thyrocytes exhibit an intrinsic defect favoring induction of apoptosis since simultaneous mild over- or under- expression of apoptosis-

promoting or inhibiting genes was detected in the thyrocytes from both strains. It remains open whether the iodolipid δ-lactone which has been isolated from porcine thyroid follicles (Dugrillon et al. 1990) as well as human thyroid tissue from patients with Graves' disease treated with high doses of iodide (Dugrillon et al. 1994) has an active role in the apoptosis mechanism operating in our system. Both iodine and δ-lactone have been reported to induce apoptosis in porcine (Langer et al. 2003) as well as human thyroid follicles (Lehmann et al. 2006) and in the thyroid carcinoma cell line B-CPAP (Gartner et al. 2010) through the mitochondrial pathway.

Thyroid epithelial cells are constantly exposed to ROS because they produce large amounts of H_20_2 required for iodination of Tg. Since high levels of ROS can lead to oxidative damage, thyrocytes have protective mechanisms to control the intracellular levels of ROS (Schweizer et al. 2008, Smyth 2003). Defects in these regulatory networks have been proposed to underlie the spontaneous thyroiditis onset in OS chickens (Bagchi et al. 1990). NOD.H2^{h4} thyrocytes, either in the basal state or after exposure to 10^{-4} M NaI *in vitro*, have previously been reported to overexpress ROS as compared to control BALB/c and B10.A thyrocytes (Sharma et al. 2008). Our data suggest that the high sensitivity of NOD.H2^{h4} thyrocytes to apoptosis, induced by low levels of NaI *in vitro*, may be, in part, due to an impairment in the up-regulation of anti-oxidant activity. 22 out of 84 oxidative stress-related genes in the array showed mild (2.01 – 3.57-fold) but significant overexpression in CBA/J thyrocytes which were resistant to apoptosis. Highest overexpression (5.77-fold) was observed with the *Tpo* gene. This contrasts with

findings from previous studies reporting either no effect of iodide treatment on *Tpo* expression (Li, Carayanniotis 2007, Leoni et al. 2008, Kawashima et al. 2013) or even downregulation of this expression (Morand et al. 2003, Muller et al. 2011) in thyroid cells or lines. The reasons for this discrepancy are unclear but may be due to the different protocols used.

Among the set of overexpressed genes encoding proteins with anti-oxidant function were several glutathione peroxidases (*Gpx2, Gpx4, Gpx7*) and peroxiredoxins (*Prdx1, Prdx2, Prdx5*) as well as *Sod1* (superoxide dismutase 1) and *Txn1* (thioredoxin 1) (Schweizer et al. 2008). Interestingly, increased expression of *Gpx2*, *Txn1* but not *Gpx4* has been previously reported in the rat PCCL3 thyroid cells after iodide treatment *in vitro* (Leoni et al. 2008, Leoni et al. 2011). Other identified genes were: a) the *Ucp2, Ucp3*, encoding the uncoupling proteins 2 and 3, located in the inner mitochondrial membrane. *Ucp2* mainly functions to attenuate mitochondrial production of ROS (Pi et al. 2010) whereas *Ucp3* seems to protect mitochondria against lipid-induced oxidative stress (Liu et al. 2013); b) *Nqo1,* (NAD(P)H dehydrogenase, quinone 1, encoding a detoxification enzyme that catalyzes reduction in quinines (Nioi et al. 2003); c) *Park7* (Parkinson's disease associated protein) encoding a protein that stabilizes Nrf2, the master regulator of antioxidant genes (Clements et al. 2006) and protects mitochondria from oxidative stress (Ma 2013); d) *Fth1* encoding the iron-storing protein ferritin that minimizes oxidative stress (Hintze, Theil 2005); e) *Serpinb1b* encoding an inhibitor of neutrophil serine proteases, with a strong anti-inflammatory activity (Polytarchou et al. 2008); f) the ROS

responsive genes *Ccl5*, encoding the chemokine (C-C motif) ligand 5 and *Psmb5*, encoding the proteasome subunit, beta type 5; g) *Mb*, encoding a protein involved in oxygen transportation with a described antioxidant function in cardiac muscle (Flogel et al. 2004); h) *Fmo2*, encoding a NADPH-dependent enzyme involved in the oxidation of various xenobiotics (Gornicka et al. 2011); i) *Txnip*, encoding a protein inhibiting the function of Txn1 and promoting induction of ROS generation (Spindel et al. 2012). Of particular interest was the upregulation of the gene *Ncf2 (p67^{phox}) (*neutrophil cytosolic factor) expression in CBA/J thyrocytes, encoding a subunit of the ROS-generating NADPH oxidase since it has been similarly reported to increase in iodide-treated BALB/c thyrocytes but not in iodide-treated NOD.H2^{h4} thyrocytes (Sharma et al. 2008). In the iodide-treated NOD.H2^{h4} group, the expression of two genes, *Gpx2* and *Nqo1*, with antioxidant function was upregulated as well as *Noxo1*, encoding for a cytosolic subunit of NADPH oxidase. Significant downregulation was observed in the expression of *Krt1*, a ROS responsive gene and *Cygb*, encoding an oxygen transporter with an antioxidant function in neuronal cells (Li et al. 2007). Since the apoptotic fate of a thyrocyte lies on the balance between ROS production and catabolism, these data imply that activation of several genes encoding proteins with anti-oxidant function plays a main role in the differential apoptosis observed in our study.

The high sensitivity of NOD.H2^{h4} thyrocytes to apoptosis induced by low concentrations of NaI *in vitro* may have important implications for the mechanisms operating in ISAT development. Apoptotic cascades in thyrocytes could be triggered by iodide and not

result simply by mononuclear cell infiltration of the gland, as described in other studies (Teng et al. 2009). The physiological iodine plasma levels in mice (Bernstein 2007, Taurog A. 1946), as well as in humans (Many et al. 1992) are estimated to be in the 10^{-7} M range and it has been reported that when the iodine intake is very high, e.g. 20 mg/day in some Japanese populations, the plasma iodine can increase 100-fold (Many et al. 1992, Nagataki 1974, Suzuki 1980). It is thus conceivable that the pro-apoptotic *in vitro* effects of NaI on NOD.H2^{h4} thyrocytes shown in the present study might reflect similar processes in ISAT when mice are placed on 0.05% NaI in water (3×10^{-3} M) for several weeks. The increased apoptotic death of NOD.H2^{h4} thyrocytes in combination with the defective clearance of apoptotic cells by macrophages that has been described in NOD mice (Maree et al. 2008, O'Brien et al. 2006) - and possibly present in NOD.H2^{h4} mice - could lead to an antigenic overload in thyroid draining LN and activation of thyroid antigen specific T cells as shown in the NOD system (Hoglund et al. 1999, Turley et al. 2003).

CHAPTER 5

The thyroxine-containing thyroglobulin peptide (a.a. 2549-2560) is a target epitope in iodide-accelerated spontaneous autoimmune thyroiditis

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5.1. Abstract

Enhanced iodide ingestion is known to accelerate the incidence and severity of disease of SAT (ISAT) in NOD.H2^{h4} mice. CD4^{+} cells are required for the development and maintenance of ISAT but their target epitopes remain unknown. In this study, we show that the previously identified Tg T-cell epitope (a.a. 2549-2560), containing T4 at a.a. position 2553 (T4p2553), induces thyroiditis as well as strong specific T and B cell responses in NOD.H2^{h4} mice. In ISAT, activated CD4^{+} T cells specific for T4p2553 are detected before the disease onset in thyroid-draining CLNC only in mice placed on iodide-rich diet and not in age-matched controls. In addition, selective enrichment of CD4^{+} IFN-γ^{+} T4p2553-specific cells is observed among CLNC and IL. T4p2553 was equally detectable on DC obtained *ex vivo* from CLNC of NaI-fed or control mice suggesting that the iodide-rich diet contributes to the activation of autoreactive cells rather than the generation of the autoantigenic epitope. Furthermore, spontaneous T4p2553-specific IgG are not detectable within the strong Tg-specific autoantibody response. These data identify for the first time a Tg T-cell epitope as a spontaneous target in ISAT.

5.2. Introduction

NOD.H2^{h4} mice are well known to develop SAT at a much higher incidence than the H-2 congenic NOD strain (Wicker et al. 1995, Hutchings et al. 1999, Braley-Mullen et al. 1999, Burek et al. 2003, Wicker 1997), while they remain free of diabetes (Podolin et al. 1993). In this model, addition of 0.05% NaI in the drinking water has been found to accelerate both the incidence and the severity of SAT (Wicker et al. 1995, Hutchings et al. 1999, Kolypetri et al. 2010, Braley-Mullen et al. 1999, Burek, Talor 2009, Wicker 1997, Rasooly et al. 1996, Teng et al. 2009), providing opportunities to study the interplay of genetics and environment in the progression of autoimmune disease. ISAT is characterized by mononuclear cell infiltration of the thyroid and strong serum IgM and IgG responses to Tg (Braley-Mullen et al. 1999, Hutchings et al. 1999, Kolypetri et al. 2010, Rasooly et al. 1996, Burek, Talor 2009, Teng et al. 2009). Immunohistochemical staining studies have suggested that the thyroid is initially infiltrated by CD4$^+$ and CD8$^+$ T cells (Verma et al. 2000, Bonita et al. 2003, Yu et al. 2001) and depletion experiments have shown that CD4$^+$ cells are required for the development and maintenance of ISAT (Hutchings et al. 1999, Braley-Mullen et al. 1999). Weak proliferative responses of thyroid-draining CLNC to Tg have been observed at various time points after the initiation of the NaI diet regimen (Kolypetri et al. 2010) but the target epitopes recognized by CD4$^+$ T cells, during the initial stages of the autoreactive cascade in ISAT, remain unknown. Since in NOD.H2^{h4} mice (K^k, A^k, E^0, D^b) functional MHC class II antigens are limited only to A^k molecules, such epitopes are expected to be recognized by A^k-restricted CD4$^+$ T cells.

Tg is the most abundant protein in the thyroid gland and encompasses many A^k-restricted T cell epitopes which can elicit EAT when administered into mice with adjuvant (Carayanniotis 2011). In NOD.H2^{h4} mice developing ISAT, we have not been previously able to detect proliferative splenic cell responses to several A^k-restricted pathogenic T cell epitopes, 56 days following the initiation of iodide supplementation (Kolypetri et al. 2010). To address the concern that thyroid antigen-specific T cells may not be detected in the periphery during later stages of the disease because they might have already migrated into the thyroid, we have in this study examined antigen-specific responses of CLNC in the initial stages of ISAT, and of IL during the course of disease. Our attention was focused on the A^k-restricted Tg peptide p2549-2560 containing T4 at position 2553 (T4p2553) because it can induce lymphocytic (Hutchings et al. 1992) as well as granulomatous EAT (Braley-Mullen, Sharp 1997) and can be seen as a target by class I-restricted cytotoxic T cells *in vitro* (Wan et al. 1998). Furthermore, T4p2553-specific T cell clones can be activated *in vivo* following mouse challenge with intact Tg (Hutchings et al. 1992). The results show that T4p2553 can be immunopathogenic in NOD.H2^{h4} mice and comprises a target epitope in ISAT recognized by spontaneously emerging $CD4^+$ T cells.

5.3. Results

5.3.1. T4p2553 is an immunopathogenic epitope in the NOD.H2^{h4} strain

To examine the immunogenicity of T4p2553 in NOD.H2^{h4}, 6-8 week old mice were s.c. challenged with 100 nmol of peptide in CFA. Nine days later inguinal, branchial and axillary LNC were pooled and tested for recall responses against various antigens *in vitro*. Strong proliferative LNC responses were detected in the presence of 1 μM (S.I. = 9) and 10 μM of T4p2553 (S.I. = 15) whereas background responses were obtained against Tg or OVA (**Figure 5.1A)**. When NOD.H2^{h4} mice were challenged with 100 μg of Tg in CFA for 9 days, weak (S.I. = 2-3) but significant proliferative LNC responses were observed against T4p2553 but the response to Tg itself or OVA remained at background levels (**Figure 5.1B**). To assess the pathogenicity of T4p2553, eight week-old NOD.H2^{h4} mice (n=10) were primed with 100 nmol T4p2553 in CFA for 3 weeks and further boosted with 50 nmol of the same peptide in IFA for 2 weeks. Control mice (n=8) were similarly sensitized with 50 μg and boosted with 25 μg OVA. Histological examination of the thyroids showed mononuclear cell infiltration in 10/10 mice of the experimental group (mean I.I. = 2.1) which was significantly higher than that observed in only 2/8 OVA-challenged controls (mean I.I. = 0.38) (**Figure 5.1C, D**). The collective data demonstrated the immunopathogenic properties of the T4p2553 T-cell epitope in NOD.H2^{h4} mice during a time interval (8-13 weeks) in which SAT is minimally present.

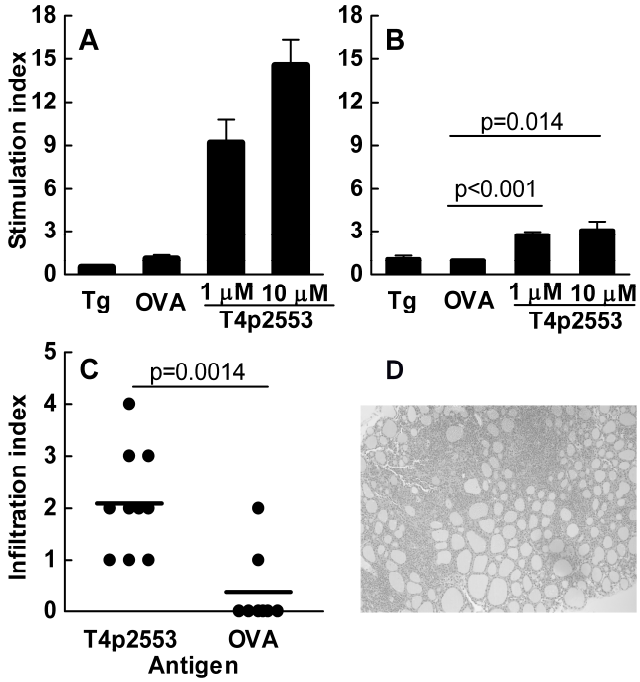

Figure 5.1

T4p2553 is an immunopathogenic epitope in NOD.H2^{h4} mice. Recall *in vitro* proliferative assays of LNC from mice s.c. challenged for 9 days with A) 100 nmol of T4p2553 in CFA (n=4), or B) 100 μg of Tg (n=4), followed by a 4 day LNC culture in the presence of the indicated antigens. Data show mean stimulation index ± SEM of triplicate wells and they are representative of two experiments; C) EAT development in NOD.H2^{h4} mice after challenge with 100 nmol T4p2553 (n=10) or 50 μg OVA (n=8), as described in Materials and Methods. Statistical significance was calculated by the Mann-Whitney test; D) Representative mononuclear cell infiltration of the thyroid (I.I. = 3) in NOD.H2^{h4} mice challenged with 100 nmol T4p2553 (Magnification, x100)

5.3.2. Accumulation of T4p2553-specific T cells in thyroid-draining CLNC and in the thyroid.

Next, we proceeded to determine whether T4p2553-specific T cells could be detected within thyroid-draining CLNC before the ISAT onset. NOD.H2^{h4} mice were placed on NaI/H_20 diet for 7, 11 and 15 days and recall *in vitro* assays against T4p2553 were performed at each time point using CLNC or splenic cells. As shown in **Figure 5.2A**, strong CLNC responses (S.I. = 8) against T4p2553, but not against Tg or OVA, were detected after 11 days of NaI intake whereas the CLNC response from control mice placed on a regular diet remained at background levels. Day 7 or day 15 responses to any of the antigens tested were undetectable (data not shown). Furthermore, day 11 T4p2553-specific responses of splenocytes were weak and not significantly different between the experimental and control groups (**Figure 5.2B**), indicating selective enrichment of antigen-specific T cells in the thyroid-draining LN and not in the periphery.

The presence of activated T4p2553-specific T cells in CLNC of iodide-fed mice was further examined by intracellular cytokine staining 12 days after the diet initiation. CD4$^+$ T cells were purified from CLNC and cultured with splenic DC as a source of APC, in the presence of antigen. Culture with T4p2553 activated a significantly higher number of IFN-γ-producing cells from CLNC of iodide-fed mice vs the controls (13.21% vs 6.22%) (**Figure 5.2C**). This difference was not evident in cultures containing the OVA peptide (322-340), as an antigen control (**Figure 5.2C**). In contrast to CLNC-derived cells, the percentage of splenic CD4$^+$ IFN-γ$^+$ cells responding to either peptide did not differ significantly between iodide-fed and control mice (**Figure 5.2D**). Lastly, 35 days after

the initiation of the dietary regimen, intracellular cytokine staining of thyroid-IL showed a small but significant difference in the percentage of $CD4^+$ IFN-γ^+ IL responding to T4p2553 vs OVA peptide (10.22% vs 7%) (**Figure 5.2E**). Overall, these data strongly suggested that an iodide-rich diet led to an early enrichment of activated T4p2553-specific cells in CLNC as well as in lymphocytic infiltrates of the thyroid during ISAT development.

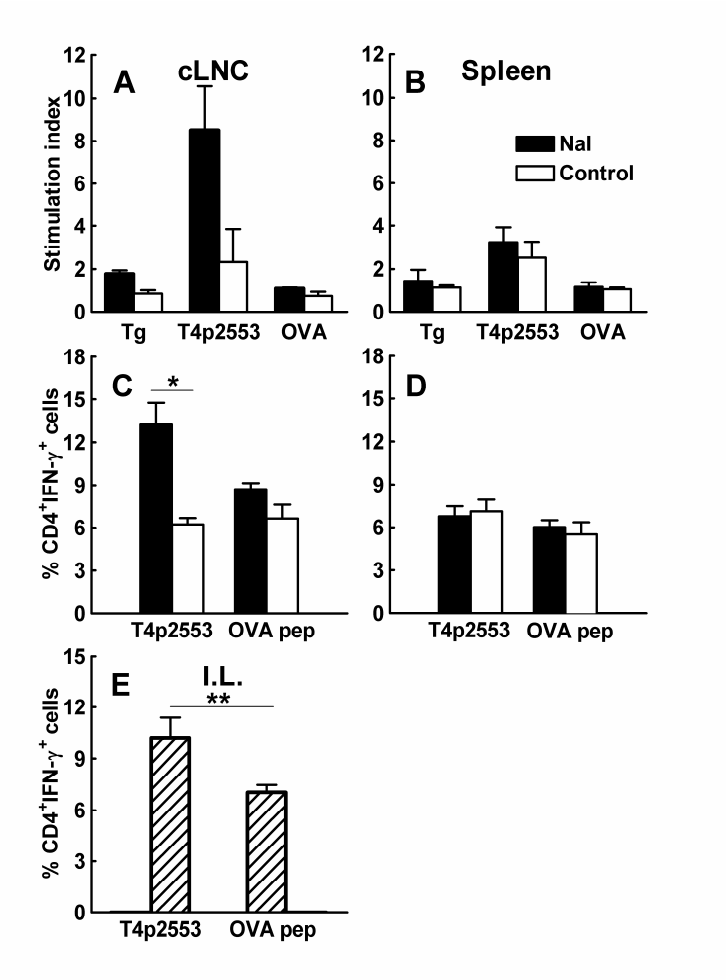

Figure 5.2

T4p2553 is a natural T-cell target in ISAT. Recall assays of A) CLNC and B) splenocytes from NaI-fed or control NOD.H2^{h4} mice (n=4) against the antigens shown, 11 days after the initiation of the dietary regimen. Antigen concentrations were: 20 μg/ml Tg, 20 μg/ml OVA or 10 μg/ml T4p2553. Cell proliferation was measured by ^{3}H-thymidine incorporation at 72 hours of culture. Data represent the mean ± SEM of quadruplicate wells from six out of 10 independent experiments in which response was detected.

Intracellular cytokine staining of $CD4^+$ cells, purified from C) CLNC and D) spleen, previously cultured (2 x 10^5 /well) with splenic $CD11c^+$ cells (4 x 10^4/well) in the presence of 20 μg/ml T4p2553 or OVA peptide for 4 days. In the last 6 hours, 5 μg/ml Brefeldin A was added to wells and the intracellular production of IFN-γ by was assessed by flow cytometry. Data are representative of three experiments. (p value= 0.0012). E) Percentages of IFN-γ^+ $CD4^+$ cells (mean ± SEM) within thyroid- IL after 35 days of enhanced NaI intake. Lymphocytes were cultured in the presence of 10 μg/ml T4p2553 or OVA peptide and 5 μg/ml Brefeldin A for 5 hours prior to staining. Data are representative of two experiments. (p value= 0.03). Flow cytometric analysis was performed on gated lymphocytes and samples were run in duplicates.

5.3.3. T4p2553 is detected on DC isolated from thyroid-draining CLNC

To test whether enhanced iodide intake would promote generation of T4p2553 on DC in lymphoid tissue, we generated KC1, a T4p2553-specific, IL-2-secreting T cell hybridoma clone, as a detection tool. The KC1 clone was selected for its sensitivity as it responded to as little as 12 nM T4p2553 in culture, but interestingly, remained unresponsive to intact Tg in the 100-200 nM range (**Figure 5.3A**). KC1 activation was strongly inhibited by an A^k-specific mAb but not by a control mAb specific for influenza NP, confirming presentation of T4p2553 in the context of the A^k molecule (**Figure 5.3B**). A low but significant activation of KC1 was equally detectable following its co-culture with *ex vivo* obtained DC from NaI-fed or control mice 12 days after the initiation of the dietary regimen (**Figure 5.3C**). These data suggested a constitutive presence of T4p2553 on DC in thyroid-draining CLNC.

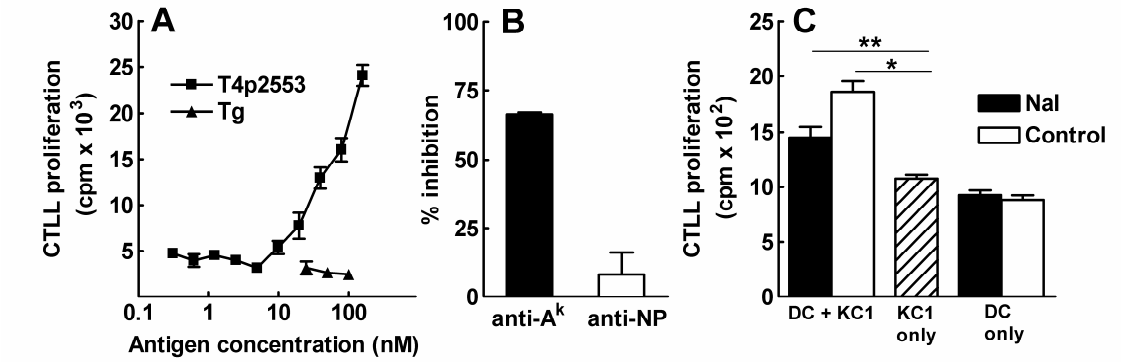

Figure 5.3

T4p2553 is constitutively present on DC from CLNC. A) T4p2553-specific reactivity of the KC1 hybridoma clone cultured (10^5/well) with the antigens shown and TA3 cells (10^5/well) as APC. Activation was measured by IL-2 secretion and the use of the IL-2 dependent line CTLL; B) Inhibition of KC1 activation by blocking mAbs specific for A^k or influenza A NP (at 10 μg/ml) in the presence of 25 nM of T4p2553 and TA3 cells as APC. Data show the mean ± SEM of triplicate wells and are representative of two experiments; C) Detection of T4p2553 on $CD11c^+$ cells purified from CLNC of iodide-fed or control mice, 12 days after the initiation of the iodide-rich diet. *Ex vivo* obtained $CD11c^+$ cells (1 x 10^4/well) were cultured with KC1 (5 x 10^4/well) for 24 hours and activation was assessed by a CTLL proliferation assay. Addition of exogenous T4p2553 yielded ~ 45,000 cpm. Data represent the mean ± SEM from 4-6 wells/group and are representative of four experiments. P values (* < 0.001, ** = 0.0003).

5.3.4. In ISAT, T4p2553-specific B cells do not contribute significantly to the Tg autoantibody repertoire

Following challenge with T4p2553, 6-8 week old NOD.H2^{h4} mice mounted strong peptide-specific as well as Tg-specific serum IgG responses, as assayed by ELISA (**Figure 5.4A**) suggesting localization of the T4p2553 epitope on the surface of the Tg molecule. The response was not due to spontaneous production of anti-Tg IgG since anti-Tg or anti-T4p2553 IgG responses were not detected in OVA-challenged age-matched mice (data not shown). In contrast, in mice developing ISAT, T4p2553-specific serological IgG responses were not detected after 30, 40, or 60 days on the NaI/water diet despite the presence of anti-Tg IgG Abs at those time intervals (**Figure 5.4B, C, D**). These results indicated that, in NOD.H2^{h4} mice developing ISAT, T4p2553-specific IgG does not comprise a detectable portion of the Tg autoantibody repertoire.

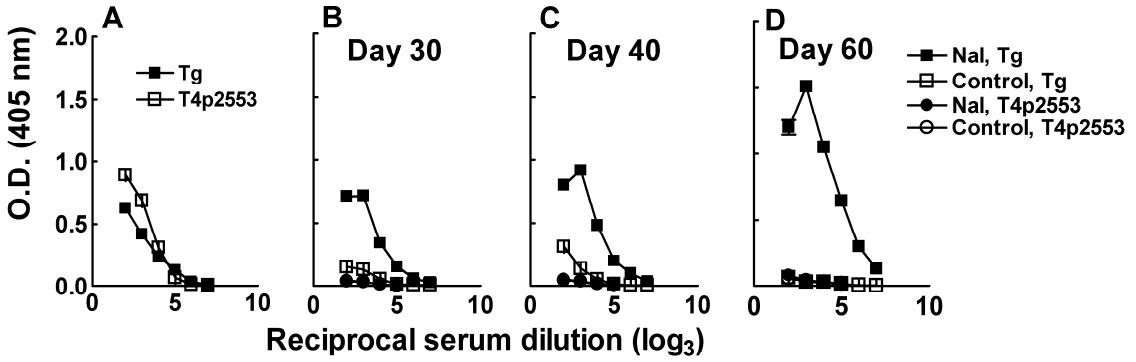

Figure 5.4

Screening for IgG responses against T4p2553 in EAT and ISAT by ELISA. Pooled sera from T4p2553-primed NOD.H2^{h4} mice (n=10) (A), or NOD.H2^{h4} mice on an iodide-rich diet for B) 30, C) 40 and D) 60 days, (n=6 per group) were tested for the presence of Tg-specific or T4p2553-specific IgG. Results are expressed as mean O.D. values of triplicate wells ± SEM after subtraction of background values from wells with no serum added.

5.4. Discussion

Our findings show that direct challenge of NOD.H2^{h4} mice with the Tg peptide T4p2553 generates EAT with 100% incidence and significant severity (mean I.I. = 2.1). Previous studies described either weak EAT induction after direct challenge with T4p2553 (Kong et al. 1995) or disease induction only after adoptive transfer of T4p2553-specific T cells (Hutchings et al. 1992, Braley-Mullen, Sharp 1997, Kong et al. 1995), perhaps reflecting differences in the protocols and the mouse strain (CBA/J vs NOD.H2^{h4}) used. *In vivo* priming with intact Tg or Tg peptide allowed for detection of subsequent T4p2553-specific, proliferative LNC responses *in vitro*, confirming earlier findings by other groups (Hutchings et al. 1992, Braley-Mullen, Sharp 1997, Kong et al. 1995). In that regard, it is surprising that deliberate challenge of NOD.H^{h4} mice with intact Tg in CFA does not yield detectable recall responses to Tg itself. This has also been observed with 52-week old NOD.H2^{h4} mice, i.e. at a time that SAT is fully developed (unpublished observations), while weak splenic Tg-specific T cell responses are obtained in ISAT (day 28 and 42 of the dietary regimen) at a time when a strong spontaneous Tg-specific IgG response is clearly evident (Kolypetri et al. 2010). These results are reminiscent of observations in diabetes where insulin-specific T cell responses are difficult to detect in NOD mice, either spontaneously or after immunization (Kaufman et al. 2001, Mohan et al. 2010, Hurtenbach, Maurer 1989) despite the presence of anti-insulin autoantibodies (Yu et al. 2000). Unresponsiveness to the intact autoantigen in this system has been attributed to possible generation of suppressor cells mediating dominant tolerance (Jaeckel et al. 2004). It is noteworthy that T4p2553-specific T cells are unlikely to

undergo negative selection pressure in the thymus because thymic cells do not synthesize T4, although they express truncated mRNA isoforms of Tg that encode this peptide sequence (Li, Carayanniotis 2005). The T4 moiety with the four bulky iodine atoms forms an integral part of the A^k-T4p2553 complex because anti-T4 Abs are known to block T-cell recognition of this peptide (Dai et al. 2005).

In this study, we report for the first time that a defined Tg epitope is a natural target of the spontaneous T-cell response in ISAT. Our previous inability to detect spontaneous splenic T cell responses to known pathogenic Tg peptides in later stages of ISAT (Kolypetri et al. 2010) prompted us to search for autoreactive T cells in thyroid-draining LN in early stages of the disease, i.e. before day 15 of the NaI/water dietary regimen, when the first signs of ISAT appear in our colony. We observed selective enrichment of T4p2553-specific, proliferative or $CD4^+$ IFN-γ^+ T cells in CLNC - but not in the spleen – only in mice receiving NaI for 11-12 days. Proliferative CLNC responses were undetectable at earlier time points (day 7) probably due to the low frequency of the peptide-specific T cells, and later time points (day 15) perhaps due to their exiting from CLNC and homing to the thyroid. Early detection of activated autoreactive T cells in CLNC, i.e. the thyroid-draining LN, is analogous to that seen in diabetes where $CD4^+$ and $CD8^+$ T cell priming in pancreatic LN occurs before the onset of insulitis in NOD mice (Hoglund et al. 1999, Zhang et al. 2002, Gagnerault et al. 2002). The presence of T4p2553-specific T cells within thyroidal infiltrates, also suggests recognition of this peptide ligand on thyrocytes – which are known to upregulate MHC class II expression

after IFN-γ treatment (Champion et al. 1991) or peptide recognition on intrathyroidal resident DC (Voorby et al. 1990).

The detection of T4p2553 on DC obtained *ex vivo* from CLNC of either iodide-fed or control mice suggests their constitutive presence on APC that is independent of iodide intake. We have previously hypothesized that T4p2553 and several other defined Tg peptides with pathogenic potential comprise part of a "cryptic self" that must be intrathyroidally expressed on resident DC under steady-state conditions since they are recognized in naïve animals by pre-activated specific effector T cells that home to the thyroid and initiate tissue damage (Carayanniotis 2003). Detection of T4p2553 on DC from CLNC suggests that either intrathyroidal DC carrying this peptide migrate to CLNC or the peptide is transferred to CLNC through the lymphatic drainage. This process may contribute to peripheral tolerance mechanisms that inactivate autoreactive T cells through anergy or active suppression (Carayanniotis 2003). Analogous findings have been reported in diabetes by Unanue's group in which $CD11c^+$ cells from pancreas or pancreatic LN were shown to constitutively present hen-egg lysozyme (HEL) peptides in mice expressing the HEL gene under the insulin promoter (Calderon et al. 2008). Constitutive expression of tissue-specific antigens such as the H^+/K^+-ATPase protein (Scheinecker et al. 2002) or foreign antigens such as the simian virus 40 T antigen (Forster, Lieberam 1996), the influenza virus hemagglutinin (Morgan et al. 1999), or OVA (Kurts et al. 1997) by DC in draining LN has been proposed to promote peripheral tolerance (Steinman et al. 2000).

Our findings indicate that high iodide intake does not promote T-cell autoreactivity to T4p2553 through enhanced generation of this peptide on DC. Analogous data have been reported with a HEL peptide-specific T cell hybridoma that was equally activated by DC from normal or streptozotoxin-treated IP-HEL mice (Calderon et al. 2008, Calderon, Unanue 2012). Furthermore, in the NOD system it has been previously shown that beta cell death, either induced by streptozotoxin or transferred by injections of dead cells in the pancreas, promotes the activation of the transgenic BDC2.5 $CD4^+$ and 8.3 $CD8^+$ T cells in pancreatic LN (Turley et al. 2003, Zhang et al. 2002). Since NOD.$H2^{h4}$ thyrocytes are highly sensitive to iodide-mediated apoptosis (Kolypetri et al. submitted for publication), it can be argued that apoptosis could trigger autoreactivity by inducing tolerogenic DC to adopt an immunogenic phenotype as has been shown in other systems (Rovere et al. 1998).

At the B cell level, T4p2553 priming induced strong peptide-specific IgG responses in NOD.$H2^{h4}$ mice, as previously seen in the CBA/J strain (Kong et al. 1995). Other groups have reported low or undetectable immunogenicity of T4p2553 in CBA hosts (Hutchings et al. 1992, Braley-Mullen, Sharp 1997) perhaps reflecting dose and route of administration differences in peptide delivery. The strong anti-Tg responses observed in T4p2553-primed NOD.$H2^{h4}$ mice were not detected in age-matched mice challenged with OVA, indicating that they are not spontaneously generated but rather reflect access of the T4p2553 site on the surface of the Tg molecule by peptide-specific IgG. In view of

this, the lack of spontaneous IgG response to T4p2553 in ISAT was surprising, demonstrating that existing T4p2553-specifc B cell clones do not contribute to the Tg autoantibody repertoire that characterizes ISAT. This suggests that Tg-specific IgG response is driven against other immunodominant B cell epitopes or by Tg fragments that lack the T4p2553 sequence. Nevertheless, T4p2553-specific B cells could bind to and internalize intact Tg, playing an APC role, as previously suggested in the NOD.H2^{h4} model (Braley-Mullen, Yu 2000).

In conclusion, this study identifies for the first time a Tg T-cell epitope as a spontaneous target in ISAT. It also highlights the fact that iodine, as a dietary element that accelerates an autoimmune process, becomes itself an integral part of a target self-epitope recognized by pathogenic T cells. Our ability to activate and monitor autoreactive T cells of a defined specificity provides a new read-out system to study effects of immune intervention in this animal model. It also generates new opportunities for the construction of TCR-transgenic mice in this model which will be useful in studies of immunoregulation.

CHAPTER 6

High salt intake does not exacerbate murine autoimmune thyroiditis

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6.1. Abstract

Recent studies have shown that high salt intake exacerbates EAE and have raised the possibility that a HSD may comprise a risk factor for autoimmune diseases in general. In this report, we have examined whether a HSD regimen could exacerbate murine autoimmune thyroiditis, including SAT in NOD.H2^{h4} mice, EAT in C57BL/6J mice challenged with Tg and EAT in CBA/J mice challenged with the Tg peptide (2549-2560). The physiological impact of high salt intake was confirmed by enhanced water consumption and suppressed aldosterone levels in all strains. However, the high salt treatment failed to significantly affect the incidence and severity of SAT or EAT or Tg-specific IgG levels, relative to control mice maintained on a normal salt diet. These data demonstrate that high salt intake does not exacerbate autoimmune thyroiditis in three experimental models indicating that a HSD is not a risk factor for all autoimmune diseases.

6.2. Introduction

Recently, two independent studies have reported that a HSD can exacerbate the severity of EAE in mice, as compared to the level of severity developed in mice fed a normal diet (Wu et al. 2013, Kleinewietfeld et al. 2013). These findings were fascinating as well as intriguing in view of the fact that data generated by the World Health Organization (WHO) do not support a direct correlation between salt intake and prevalence of multiple sclerosis in humans (Croxford et al. 2013, Elliott, Brown). Nevertheless, the reported dramatic exacerbation of EAE in mice on HSD, has highlighted the possibility that increased salt intake may aggravate clinical symptoms in individuals genetically predisposed to MS and most importantly, has raised the question whether HSD might represent a risk factor for the development of other autoimmune diseases.

In this report, we have examined potential HSD effects on the development of autoimmune thyroiditis in mice. The experimental design was made to monitor incidence and severity of disease in three mouse models that have been used extensively to study pathogenetic mechanisms analogous to those operating in HT in humans. In the first group, we used NOD.H2^{h4} mice which are known to develop SAT with age (Kolypetri et al. 2010, Braley-Mullen et al. 1999, Burek et al. 2003). In the second and third groups, we asked whether mild forms of EAT, induced after challenge with thyroid antigen in adjuvant, could be exacerbated by concomitant high salt intake. To this purpose, we challenged C57BL/6 mice with Tg and CBA/J mice, with the known pathogenic Tg peptide (2549 – 2560) containing T4 at amino acid position 2553 ((T4)2553) (Champion

et al. 1991, Hutchings et al. 1992). We also monitored Tg-specific serum IgG responses as an index of autoreactivity in all strains.

6.3. Results and Discussion

Following placement of NOD.H2^{h4}, C57BL/6 and CBA/J mice on HSD (n=10, per strain) or control diet (n=10 per strain), water intake was monitored weekly over the 5-week observation period, as an index of response to the dietary regimen (Veress et al. 1995). Mice on HSD showed a highly significant increase of water intake with average (mean ± S.E.) daily consumption rates 15.5 ± 0.71, 9.1 ± 0.3 and 7.3 ± 0.2 g/mouse for NOD.H2^{h4}, C57BL/6 and CBA/J strains, respectively, while control values ranged between 3.2 – 4.3 g/day **(Figure 6.1a)**. At the end of the study period, the effectiveness of the HSD was further assessed by measurement of serum aldosterone levels to check for the high salt-induced suppression reported by others (Kim et al. 2008, Makhanova et al. 2008). A profound suppression of serum aldosterone was observed in the experimental vs. the control groups in all strains: 13 vs. 381 pg/ml in NOD.H2^{h4}, 13 vs. 254 pg/ml in C57BL/6, and 21 vs. 386 pg/ml in CBA/J mice (**Figure 6.1b**).

NOD.H2^{h4} mice, developed by Dr. L. Wicker and colleagues for studies on diabetes (Podolin et al. 1993), were chosen as a first animal model for our study because it is the only mouse strain known to develop SAT at an increasing incidence and severity with age. In our colony, the first signs of spontaneous thyroiditis appear in 12-14 week-old male or female mice (Kolypetri et al. 2010) and at 55 weeks of age, practically all mice exhibit SAT of advanced severity and seropositivity for Tg-specific IgG (unpublished observations). While thyroiditis incidence and severity may differ among various colonies, analogous age-dependent effects on SAT have been reported by others (Braley-Mullen et al. 1999, Burek et al. 2003). In this report, our aim was to test whether HSD,

initiated at the early stages of SAT, would influence the development of disease. As shown in **Table 6.1**, placement of 12 week-old NOD.H2^{h4} mice on HSD for five weeks did not significantly raise the incidence (6/10 vs. 9/10) or severity (I.I.= 1.3 vs. 1.6) of SAT vs. those of the controls. In addition, there was no significant increase in the number of mice spontaneously developing IgG antibodies to Tg (**Figure 6.1c**).

In a second series of experiments, we followed the experimental design of Wu et al (Wu et al. 2013) and Kleinewietfeld et al (Kleinewietfeld et al. 2013) by placing C57BL/6 mice on the same high salt regimen (4% NaCl in chow, 1% NaCl in drinking water) that exacerbates EAE in this strain and a similar period of observation (5 weeks). An added attractive feature was that, in the field of EAT, this strain is well known to develop relatively low levels of thyroiditis after challenge with Tg in adjuvant (Vladutiu, Rose 1971), thus favoring investigation into possible enhancing effects of high salt intake on pathology in this model. The results showed that a dietary regimen of high salt, continued throughout the period of antigenic challenge, did not increase the EAT incidence (9/10 vs. 6/10) or mean severity (I.I. = 1.2 vs. 0.9) vs. that of the controls (**Table 6.1**). Moreover, strong, but not significantly different Tg-specific IgG responses were detected in both the high salt and control groups (**Figure 6.1c**).

In a third experimental model, we challenged the EAT-susceptible strain CBA/J (Vladutiu, Rose 1971) with the 12-mer T-cell epitope (T4)2553 of Tg (Champion et al. 1991, Hutchings et al. 1992) at doses that are mildly pathogenic. Mice in the experimental group were placed on HSD during the 5 week induction phase of EAT. The

high salt regimen had again no significant effect either on the incidence (6/10 vs. 4/10) or mean severity (I.I. = 0.9 vs. 0.5) of disease (**Table 6.1**). The (T4)2553 peptide elicited a Tg-specific IgG response of similar relative strength in both groups, which was, as expected, lower than that obtained in C57BL/6 mice immunized with intact Tg (**Figure 6.1c**).

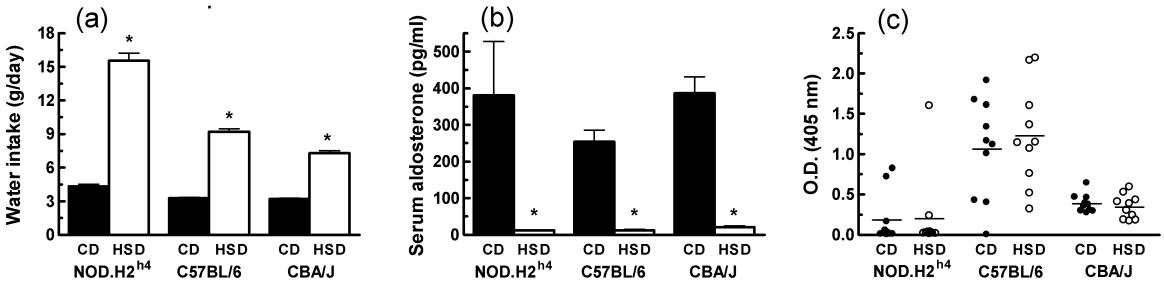

Figure 6.1

Effects of HSD vs. control diet (CD) on physiological and immunological parameters in mice developing autoimmune thyroiditis. (a) Water intake (g/day) in mice placed on HSD or CD for 5 weeks expressed as mean ± SE of values (n=10 per group). Asterisks denote statistical significance assessed by a t-test (p values for all groups $<10^{-7}$). (b) Serum aldosterone levels measured by RIA at the end of 5-week observation period. Five samples were run for each experimental and control group, with each sample being pooled from two randomly selected mice within a group. The data represent the mean ± SE values in each group and asterisks denote statistical significance assessed by a t-test ($p<0.036$ for NOD.H2^{h4}; $p<10^{-4}$ for C57BL/6 and CBA/J). (c) ELISA-based Tg-specific IgG responses from individual mouse sera, observed spontaneously (NOD.H2^{h4} mice) or after antigenic challenge as described in Materials and Methods (C57BL/6 and CBA/J mice). Serum dilutions for the assay were 1:25 for NOD.H2^{h4}, 1:50 for C57BL/6 and 1:100 for CBA/J mice. Data represent the mean ± S.D. optical density values of triplicate wells.

Table 6.1. Thyroiditis incidence and severity in NOD.H2^{h4}, C57BL/6 and CBA/J mice

Strain	NaCl [a]	Immunizing antigen	Thyroiditis						p value [b]
			Incidence	Severity (score)				Mean	
				0	1	2	3	4	
[c] NOD.H2^{h4}	+	-	6/10	4	1	4		1	1.3	0.63
	-	-	9/10	1	5	2	1	1	1.6	
[d] C57BL6/J	+	Tg	9/10	1	7	1	1		1.2	0.39
	-	Tg	6/10	4	4	1	1		0.9	
[e] CBA/J	+	T4p2553	6/10	4	3	3			0.9	0.35
	-	T4p2553	4/10	6	3	1			0.5	

[a)] Mice received normal chow and water *ad libitum* or chow containing 4% NaCl and water containing 1% NaCl *ad libitum*, over a 5 week period, as described in section 2.1

[b)] Statistical significance was assessed by the non-parametric Mann-Whitney test.

[c)] Each group consisted of 5 female and five male 12-wk old thyroiditis-prone NOD.H2^{h4} mice.

[d)] Female C57BL/6J mice (n= 10 per group) were s.c. challenged with 100 μg Tg in CFA and the experimental group was simultaneously placed on HSD. Three weeks later, all mice received 50 μg Tg in IFA. EAT was determined 5 weeks after the initial antigenic challenge.

[e)] Female CBA/J mice (n = 10 per group) were s.c. challenged s.c. with 100 nmol of T4p2553 peptide in CFA and the experimental group was simultaneously placed on HSD. Three weeks later, all mice received 50 nmol of the same peptide in IFA. EAT was determined 5 weeks after the initial antigenic challenge.

The focus of the current work was to examine the thesis that there is a tangible link between HSD and autoimmune disease other than EAE, using mouse models of autoimmune thyroiditis. The data provided demonstrate strongly that dietary salt does not promote either antigen-induced or spontaneous thyroid autoimmune disease. The report that C57BL/6 mice on high salt intake develop enhanced EAE after challenge with an encephalitogenic MOG peptide in CFA (Wu et al. 2013, Kleinewietfeld et al. 2013) is in apparent contrast with our finding that delivery of a thyroiditogenic molecule (Tg) to the same strain via the same route and under the same high salt conditions does not lead to enhanced EAT. To the extent that the studies of Wu et al. (Wu et al. 2013), and Kleinewietfeld et al. (Kleinewietfeld et al. 2013) have assigned a pivotal role to pathogenic Th17 cells for the exacerbation of EAE, our data suggest that either a) Th17 cells are not as critical in EAT/SAT development as in EAE, or b) additional pleiotropic physiological, hormonal and metabolic factors, triggered by HSD, mediate EAE exacerbation in a unique manner that is not necessarily duplicated in EAT/SAT.

EAT and SAT are well known to be mediated by Th1 cells (Yu et al. 2002) – but a role for Th17 cells in pathogenesis remains possible. Interleukin 17 (IL-17)$^{-/-}$ NOD.H2^{h4} mice have been reported to exhibit reduced iodide-accelerated SAT and diminished Tg-specific titers as compared to WT controls, while intrathyroidal cytokine mRNA analysis has suggested the presence of both Th1 and Th17 cells in thyroidal lymphocyte infiltrates of WT NOD.H2^{h4} mice (Horie et al. 2009). In humans, an increased frequency of Th17 cells has been reported in the peripheral blood of patients with HT (Figueroa-Vega et al. 2010,

Qin et al. 2012, Shi et al. 2010) or autoimmune thyroid disease (AITD) (HT and Graves' disease) (Nanba et al. 2009, Hayashi et al. 2009) and it has been recently suggested that T cell derived-leptin may contribute to the increased frequency of Th17 cells in HT (Wang et al. 2013). Associations of single nucleotide polymorphisms in AITD have been described for the IL-23R gene in Japanese (Ban et al. 2009) and for the IL-17A and IL-17F genes in Chinese populations (Yan et al. 2012). However, since IL-17 is produced by several cell types including $CD8^+$ cells and γδ T cells (Hirota et al. 2011, Ivanov et al. 2006, O'Shea, Jones 2013), association data from both mouse and human studies may not necessarily reflect a cause-and-effect relationship between Th17 cells and thyroid pathology. Lastly, it is noteworthy that even in the pivotal EAE studies (Wu et al. 2013, Kleinewietfeld et al. 2013), exacerbating effects of HSD on disease were demonstrable in WT mice during the first twenty days of the dietary regimen. When monitoring continued until day 25 in one of the studies, no significant differences between HSD and control groups were found (Wu et al. 2013). In the work presented here, while no significant effects on HSD on EAT were observed using a well-established 5 wk disease induction protocol, we cannot exclude the possibility that high salt intake might have had transient, non-sustained effects on EAT that were not apparent after 5 weeks. Also, we cannot exclude the possibility that the effects of high salt intake on thyroiditis could reach statistical significance if a larger sample size is analyzed.

Regardless of the mechanism involved, our data clearly demonstrate that excess salt in diet should not be expected to be an environmental risk factor that broadly influences

induction of autoimmune disease. Enhanced salt intake is accompanied by fluid, ionic and hormonal changes including suppression of the renin-angiotensin system (de la Sierra et al. 1996, van der Meer, Netea 2013) which could differentially influence autoimmune responses depending on the anatomical site and metabolic status of the target organ. The planning of clinical trials to investigate low-salt diets for treating or preventing autoimmune disease should proceed with caution, taking into account relevant data from diverse animal model studies.

SUMMARY AND FUTURE DIRECTIONS

The thesis describes studies on the immunoregulation of autoimmune thyroiditis using murine models of EAT, SAT and ISAT. In this section, a brief summary of, and future directions related to each chapter are outlined.

Chapter 3

We examined whether immunopathogenic and/or immunodominant Tg epitopes could be located adjacent to proteolytic sites using the EAT model. We predicted possible cathepsin B, D and L cleavage sites in mTg and we identified H2-A^k binding motifs adjacent to the proteolytic sites. Peptides encompassing these motifs were synthesized and two of them, p2369 (a.a. 2369-2380) and p2439 (a.a. 2439-2450) located close to cathepsin L cleavage sites, were found to be immunopathogenic but not immunodominant in CBA/J mice. This finding raised the number of pathogenic, cryptic peptides to 27.

Hypothesis #1: Tg epitopes, previously defined as "cryptic", become dominant after processing of Tg fragments by DC.

The reasons behind the inability to identify a dominant Tg epitope are unknown. A plausible hypothesis is that Tg epitopes, previously identified as cryptic, may become immunodominant after processing of Tg fragments rather than intact Tg. To test this premise, intact Tg will be partially digested with cathepsins B and L and the reaction will

be irreversibly blocked by a cysteine protease inhibitor. Tg fragmentation will be confirmed by SDS-PAGE. The protein solution will be then subjected to reverse phase HPLC and the fractions will be pooled and concentrated. BM-derived DC from CBA/J mice will be incubated with titrating amounts of the Tg fragment concentrate or intact Tg (as negative control) or a known free cryptic Tg peptide such as T4p2553 (as positive control). Peptide-primed T cells or peptide-specific T cell hybridomas will be added to the wells and their activation will be used as a readout system. As additional control, chloroquine-treated DC will be used to exclude the possibility that the peptide binds exogenously to the MHC molecules. If our hypothesis is correct, we expect to detect T activation in the presence of the Tg fragment concentrate but not in the presence of intact Tg.

Pitfalls: Inability to detect a T cell response could be attributed to: a) wrong choice of epitope to test the concept. In this case, the experiment can be repeated with other kwown pathogenic Tg peptides; b) epitopes generated after processing of Tg fragments may be expressed at levels below the required T cell activation threshold.

Hypothesis #2: Tg epitopes, previously defined as “cryptic”, are constitutively generated and expressed on DC within the thyroid gland.

Previous studies have shown that Tg peptide-activated T cells selectively home to the thyroid after direct challenge of mice with peptide in CFA or after adoptive transfers to naïve recipients. These observations made us hypothesize that cryptic Tg peptides are constitutively generated and expressed on intrathyroidal DC. To address this concept,

intrathyroidal DC will be sorted from SJL fresh thyrocyte suspensions via anti-CD11c Ab staining. Then, DC will be cultured with Tg peptide-primed T cells or Tg peptide-specific T cell hybridomas without the addition of exogenous peptide for 72 hours. The peptide used in this design will be a randomly chosen Tg cryptic epitope in the first experiment but the presence of 4 more cryptic epitopes will be examined using the similar design. As additional control, in some wells exogenous peptide will be added. T cell proliferation will be assessed by thymidine uptake and the production of IL-2 and IFN-γ in the supernatant will be examined by sandwich ELISA. T cell hybridoma activation will be assessed by the proliferation of CTLL cells. If we detect significant T cell proliferation or activation in the absence of exogenous peptide that would indicate that this cryptic peptide was generated and expressed on the surface of DC.

Pitfalls: a) A large number of animals, as thyroid donors, will be required because the intrathyroidal DC yield is low; b) T-cell activation may not be observed if Tg peptides are endogenously generated on DC at levels insufficient to trigger a T cell response *in vitro.*

Chapter 4

We showed that iodide-treated NOD.H2^{h4} thyrocytes undergo apoptosis at significantly higher levels than CBA/J thyrocytes and that was associated with an impaired control of oxidative stress mechanisms. Since the majority of the molecular events involved in iodide-mediated apoptosis remain unknown, the following experiments could be performed.

Hypothesis #1: The mitochondrial pathway is involved in the early stages of iodide-induced apoptosis of NOD.H2^{h4} thyrocytes.

Previous studies have reported that mitochondria are involved in the early events of apoptosis triggered by high iodide doses in FRTL-5 cells (Yao et al. 2012). To examine whether this organelle participates in the initial events in iodide-induced apoptosis of NOD.H2^{h4} thyrocytes, we will perform a kinetic analysis of the production of superoxide as well as the release of apoptogenic factors from mitochondria. Thyrocytes from NOD.H2^{h4} and CBA/J mice will be cultured for 9 days *in vitro*. On day 9, 4 x 10^{-6} M NaI or medium will be added to the wells and 2, 4, 6 and 8 hours later, the cells will be stained with MitoSOX Red, a mitochondrial superoxide indicator and they will be analyzed by flow cytometry. As additional control, cells will be stained only with propidium iodide to verify the integrity of the membrane. If our hypothesis is correct, we expect to detect an increased production of mitochondrial superoxide after 2-8 hours in iodide-treated NOD.H2^{h4} thyrocytes compared to all control groups. Furthermore, we will examine the release of the proteins cytochrome c and apoptosis-inducing factor in the cytoplasm. Cells will be treated with or without 4 x 10^{-6} M NaI for 2, 4, 6 and 8 hours. Then, thyrocytes will be lysed and centrifuged to separate the cytosolic and mitochondrial fractions. The concentration of cytochrome c in the cytosolic and mitochondrial fractions will be estimated by an ELISA kit while the presence of apoptosis-inducing factor will be examined by Western blot analysis. If our hypothesis is correct, we expect increased concentrations of one or both proteins to be detected in the

cytoplasm during the first 2-8 hours after iodide treatment. The data will indicate the involvement of mitochondria during early stages of iodide-mediated apoptosis but they will not answer whether the mitochondrial changes are the cause or the effect of the apoptotic cascade in iodide-treated thyrocytes.

Hypothesis #2: NOD.H2^{h4} thyrocytes are highly susceptible to iodide-mediated apoptosis due to defective expression and/or function of the transcription factor Nrf-2.

The nuclear factor erythroid 2 (Nrf-2) is a basic region-leucine zipper-type protein that acts as the master regulator of genes involved in anti-oxidant defense mechanisms (Uruno et al. 2013). Under normal conditions, Nrf-2 tightly interacts with the adaptor protein Keap-1 in the cytoplasm, facilitating ubiquitination and degradation of Nrf-2 by proteasomes (Ma 2013). Under stress conditions, electrophiles or ROS can interact and modify the cysteine residues of Keap1, leading to conformational changes in the Keap1-Nrf-2 complex. These changes allow Nrf-2 to escape cytosolic degradation and translocate to the nucleus where it induces the expression of itself as well as of several genes with anti-oxidant function or ROS metabolism (Ma 2013). The Nrf-2 anti-oxidant signaling pathway has been shown to have a critical role in the survival of mammary epithelial cells (Gorrini et al. 2013) and protect pancreatic β-cells from ROS-mediated damage (Yagishita et al. 2014).

In **Figure 4.4**, the expression of 10 Nrf-2 regulated genes (*Sod1, Gpx2, 7, 4, Prx1, 2, 5, Park7, Fth1* and *Nqo1*) (Ma 2013) was significantly upregulated in CBA/J thyrocytes

whereas only 2 Nrf-2 regulated genes (*Gpx2*, *Nqo1*) had higher expression in NOD.H2^{h4} thyrocytes after iodide treatment. To examine whether there is a defect in the expression levels of Nrf-2 in NOD.H2^{h4} mice, NOD.H2^{h4} and CBA/J primary thyrocytes will be cultured for 9 days. On day 9, cells will be treated with 4 x 10^{-6} M NaI or medium for 24 hours. Then, mRNA will be isolated from all groups and the expression levels of the Nrf-2 will be analyzed by qPCR. If there is a defect at the expression level, then we expect to detect significantly higher levels of Nrf-2 mRNA expression in the CBA/J vs the NOD.H2^{h4} thyrocytes after iodide treatment. Our findings will also be examined at the protein level by Western blot analysis.

To examine whether there is a defect in the regulation of the Nrf-2 protein, its interaction with the protein Keap-1 will be examined under normal and stress conditions by immunofluorescence staining. Thyrocytes from both strains will be cultured with or without 4 x 10^{-6} M NaI for 24 hours and then cells will be fixed, stained with a FITC-conjugated anti-Nrf-2, PE-conjugated anti-Keap1 mAb and DAPI and they will be analysed by immunofluorescence microscopy. If the regulation of the Nrf-2 protein in the iodide-treated NOD.H2^{h4} group is defective then we expect to detect the two proteins co-localized in the cytoplasm whereas in the iodide-treated CBA/J group the Keap1 protein will be in the cytoplasm and the Nrf-2 will have been translocated to the nucleus. The pitfall in this approach is that Nrf-2 may translocate to the nucleus in iodide-treated NOD.H2^{h4} thyrocytes but still have an impaired capacity to regulate the transcription of genes due to strain-specific polymorphisms in the Nrf-2 protein.

Hypothesis #3: *Ex vivo* detection of iodide-induced apoptosis of NOD.H2^{h4} thyrocytes.

Previous studies in the NOD mouse have highlighted that spontaneous apoptosis in pancreatic sections has been difficult to detect due to the rapid clearance of apoptotic cells as well as their low frequency even at peak times (4-13%) (Mathis et al. 2001). To examine if in our model, administration of iodide-rich diet could cause a significant increase in the number of apoptotic thyrocytes within the gland to allow their detection, we will perform the following experiment. Six to eight week old NOD.H2^{h4} and CBA/J mice will be placed on normal or 0.05% NaI containing water for 2-10 days and a kinetic analysis of apoptosis on a daily basis will be performed using the NIR-FLIVO^{TM} 690. FLIVO^{TM} is a cell-permeable tracer that circulates throughout the body and binds irreversibly to activated caspases. Apoptotic cells can be visualized using live animals in a whole animal imager or thyroid glands can be isolated and cell suspensions analyzed by fluorescence microscopy or flow cytometry to quantify the number of apoptotic cells. If our hypothesis is correct, we expect to detect a significant number of apoptotic thyrocytes in glands from iodide-treated NOD.H2^{h4} mice compared to all control groups.

Pitfalls: The difference in the number of apoptotic cells between the iodide-fed and control group may be small and not reach statistical significance.

Chapter 5

We have reported that the Tg peptide T4p2553 is a natural T cell target recognized at early stages - before ISAT onset - in NOD.H2^{h4} mice. This observation as well as the generation of the T4p2553-specific T-cell hybridoma clone KC1 are quite advantageous for further studies in the NOD.H2^{h4} model. Cloning of the VJ and VDJ sequences of the TCR of the KC1 hybridoma clone will allow us to construct the KC1 TCR transgenic mouse in the NOD.H2^{h4} background. The existence of such a transgenic mouse will help us overcome the problem of the low frequency of ag-specific T cells and facilitate the design of our experiments as in the NOD system with the BDC2.5 and 8.3 TCR transgenic mice.

Our data have also shown that even though T4p2553 was detected on the surface of CLN DC regardless of iodide ingestion, spontaneous T cell activation against the peptide was recorded only in CLNC of mice receiving iodide-rich diet. This observation made us speculate that a high rate of iodide-induced apoptosis of thyrocytes (as described in Chapter 4) inside the gland may have altered the tolerogenic function of DC to immunogenic leading to T cell activation in the draining lymph nodes. To shed more light on the events triggered by iodide that lead to T cell activation in CLN, we will test the following hypotheses:

Hypothesis #1: Iodide-mediated apoptosis of thyrocytes abrogates the tolerogenic **phenotype** of tissue-draining migratory DC.

The tissue-draining LN contain two groups of DC. The LN-resident DC - plasmacytoid, $CD8^+$, $CD8^-$ DC- as well as the tissue draining migratory DC - $CD103^+$ and $CD11b^+$ DC that have migrated to the nodes from the drained tissue (Miller et al. 2012). Recent studies have shown that the tissue-draining, migratory DC, have a crucial role in peripheral tolerance induction through the activation of Treg cells (Yamazaki et al. 2008, Idoyaga et al. 2013). Interestingly, the molecular signature of this DC subset - which is not shared with LN-resident DC - has been identified by the Immunological Genome Consortium (Miller et al. 2012). Under steady state, migratory DC express immune response-dampening molecules like *Itgb8* (encoding TGF-β-activating integrin β8) and *Socs2* (encoding a TLR-responsive molecule that regulates the release of cytokines from DCs) whereas under inflammatory conditions they express *Cxcl10, Ccl3, Cxcl9, Il6, Il1b, and Il1a* (Miller et al. 2012).

To test hypothesis #1, we will follow an *in vitro* and an *in vivo* approach. In the *in vitro* experiment, $NOD.H2^{h4}$ thyrocytes will be cultured with titrating concentrations of NaI for 24 hours. During this incubation period, cells will become apoptotic as shown in Chapter 4. Then, thyrocytes will be washed and cultured for 1, 2 and 3 days with syngeneic migratory DCs (or resident DC as controls) purified from pooled LN (cervical, brachial, axillary, inguinal) as described by (Miller et al. 2012). Additional control groups will include migratory or resident DC cultured either with medium alone (steady

state DC) or with poly (I:C) (activated DC). Subsequently, $CD11c^+$ cells will be recovered and their cDNA will be analyzed by real-time RT-PCR using appropriate primers for the above-mentioned genes. If our hypothesis is correct, we expect that migratory DC incubated with apoptotic thyrocytes will reduce expression of *Itgb8* and *Socs2* while they will increase expression of genes encoding the inflammatory molecules described above.

In the *in vivo* approach, six to eight week old NOD.$H2^{h4}$ mice will be placed on NaI containing water or normal water for 7-9 days i.e. at a time point before the onset of ISAT. Migratory DC and control resident DC from CLNC will be isolated and mRNA will be used for Real-Time PCR analysis. Corresponding DCs from thyroid-non draining LN (inguinal) will be used as controls. If our hypothesis is correct, we expect that only migratory DC from CLNC of mice on iodide-rich diet will have reduced expression of *Itgb8* and *Socs2* and increased expression of genes encoding the inflammatory molecules described above.

<u>Hypothesis #2: The migratory $CD11b^+$ DC is the APC subset carrying the T4p2553 from the thyroid to CLN.</u>

Previous studies in the NOD mouse have suggested that β cell antigens are transported from the pancreas to the pancreatic LN by DC before insulitis onset. Interestingly, the relevant DC subset was characterized as $CD11c^+CD11b^+CD8\alpha^-$ (Turley et al. 2003). In our system, the majority of intrathyroidal DC have been characterized as

$CD11c^{low}CD11b^{+}CD8\alpha^{-}$ cells (Klein, Wang 2004). To examine whether this DC subset carries the T4p2553 peptide from the thyroid to the CLN, migratory $CD103^{+}$, migratory $CD11b^{+}$, resident $CD8\alpha^{+}$, $CD8\alpha^{-}$ DC subsets will be purified from the CLN of 6-8 week old NOD.$H2^{h4}$ mice. These subsets of DC will be cultured for 24 hours with the T4p2553-specific KC1 T cell hybridoma without addition of exogenous T4p2553. Activation of the hybridoma will be assessed by the IL-2 production in a CTLL assay. *Ex vivo* obtained migratory and resident DC subsets from thyroid-non draining (inguinal) LN will be used as additional controls. In some cultures, exogenous p2495 will also be added as positive control. If our hypothesis is correct, we expect to detect significant T cell activation only in the presence of migratory $CD11b^{+}$ cell from CLN. These data will indicate that this subset only expresses the T4p2553 endogenously on its surface. These data would also imply that this is the DC subset transporting the peptide from the thyroid to CLN. The pitfall in this design is that if T4p2553 is transferred via the lymph to the CLN and not by a specific DC subset then it should be present in resident DC as well.

Hypothesis #3: Tolerogenic semimature DC pulsed with T4p2553 can suppress ISAT

Previous studies from our laboratory have shown that tolerogenic semimature DC pulsed with Tg can activate $CD4^{+}CD25^{+}$ Treg cells and suppress EAT (Verginis et al. 2005). Studies with NOD mice have shown that tolerogenic DC, presenting a single autoantigenic peptide (mimotope), can activate $CD4^{+}CD25^{+}$ Treg cells and suppress autoimmune diabetes (Tarbell et al. 2004). To examine whether tolerogenic DC pulsed

with only one Tg epitope, the T4p2553, can suppress ISAT via activation of Treg in NOD.H2^{h4} mice, the following experimental design will be followed.

NOD.H2^{h4} bone marrow cells will be cultured for 9 days in the presence of GM-CSF and TNF-α (40 ng/ml) or LPS (1 μg/ml) or medium will be added for an additional 24 hours of culture. Non adherent cells will be examined for DC generation based on CD11c expression, and they will be monitored for phenotypic expression of MHC class II, CD80, CD86, and CD40 molecules. Sandwich ELISA in DC culture supernatants will test for the levels of IL-12, IL-1β, and IL-6 secretion. TNF-α treated DC would be expected to acquire a semi-mature phenotype, i.e. increased expression of MHC class II, CD80, CD86, and CD40 molecules compared to DC generated only with GM-CSF as well as low levels of IL-12, IL-1β, and IL-6 secretion compared to LPS-treated DC.

To assay the tolerogenic function of TNF-α treated DC in vivo, DC will be pulsed with T4p2553, intact Tg or ovalbumin (antigen control) for 6 hours, treated with TNF-α for 24 hours, and then adoptively transferred i.v. into naïve 6-8 wk old NOD.H2^{h4} mice in three injections (days 0, 2 and 4, 3x10^6 cells/mouse). On day 0, mice (n= 10) will be placed on NaI/$H_2$0 for 28 days to develop ISAT while a control group will be placed on normal diet. Additional controls will be CBA/J mice that receive T4p2553-pulsed tolerogenic DC to suppress T4p2553-induced or Tg-induced EAT, as previously (Verginis et al. 2005). If TNF-α-treated, semi-mature Tg-pulsed or T4p2553-pulsed DC turn out to

suppress ISAT in NOD.H2^{h4} mice, this will support the view that Tg-specific and/or T4p2553-specific Treg cells exist in this strain and can be expanded by this protocol.

To seek evidence that CD4^{+}CD25^{+} T cells from mice injected with Tg- or T4p2553-pulsed DC have the characteristics of the Treg cell subset, the expression levels of CD25, CD62L, Foxp3 and glucocorticoid-induced TNF receptor (GITR) in CD4^{+}CD25^{+} and CD4^{+}CD25^{-} T cells will be examined by flow cytometry. Treg cell function will be tested by mixing experiments *in vivo* or *in vitro*. The induced CD4^{+}CD25^{+}Foxp3^{+} cells will be purified by flow cytometry and their putative suppressive function will be tested *in vitro* as described (Yamazaki et al. 2008). CFSE-labelled responder CD4^{+} CD25^{-} T cells from WT NOD.H2^{h4} mice will be stimulated with anti-CD3 mAb and spleen APCs. In these wells, titrated numbers of Treg cells – purified from mice injected with Tg, T4p2553 or ova pulsed DC - will be added and 3 days later, CFSE dilution will be analyzed by flow cytometry. Evidence of suppressive function *in vitro* will prompt the design of adoptive transfer experiments to ascertain whether this Treg population from Tg- and/or T4p2553-pulsed DC can prevent ISAT development. To this end, CD4^{+}CD25^{+}Foxp3^{+} cells will be purified from mice injected with Tg, T4p2553 or OVA pulsed DC. Treg cells or PBS will be transferred to naïve recipients and one day later mice will start receiving iodide-rich diet for 28 days. Thyroids will be removed at the end of this 28 day period. Additional control groups will include mice that will receive Treg cells from all groups but they will be administered normal water for 28 days.

Hypothesis #4: Spontaneous T cell activation against other Tg epitopes can be detected in CLN during the initial stages of ISAT.

Previous studies in EAT have identified several pathogenic A^k-binding Tg peptides in CBA/J mice. To examine whether these peptides - I-p117, I-p179, I-p304, I-p1931, I-p2529, I-p2540 and their non-iodinated analogs (Li, Carayanniotis 2006, Li et al. 2007) as well as the peptides p306, p1579, p1826, p2102, and p2596 (Verginis et al. 2002) - are recognized by $CD4^+$ CLNC after NaI admininistration in NOD.$H2^{h4}$ mice, the following experiment will be performed. $CD4^+$ cells will be purified from CLN and splenocytes 7, 11 and 15 days after initiation of the iodide-rich diet and their proliferative responses against the peptide panel described above will be tested. Splenic $CD11c^+$ cells will be used as APC. $CD4^+$ cells from CLN and splenocytes of mice on a normal water diet will be used as controls. If our hypothesis is correct, we expect to detect significant proliferative responses against other Tg epitopes only in CLN of iodide treated mice.

Chapter 6

We examined whether HSD exacerbates autoimmune thyroiditis using three animal models of the disease. Our data showed that there was no significant difference in the incidence and severity of thyroiditis between animals on high salt and normal diet as previously shown in the EAE model (Wu et al. 2013, Kleinewietfeld et al. 2013). This study however, raised the question of whether Th17 cells have a pathogenic role in thyroiditis, a concept that has not been studied extensively before. To examine this concept, we will make use of a TCR transgenic SJL mouse model specific for the

pathogenic Tg peptide p2495 (LINRAKAVK) (a.a. 2495-2503) that was generated in our laboratory. The p2495-specific TCR was obtained from the A^s-restricted T cell hybridoma clone 9.13 which secretes IL-2 upon specific activation with p2495. The VJ and VDJ sequences of the 9.13 TCR were cloned and microinjected into fertilized (B6 x SJL) F1 oocytes. Successful production of a fertile founder was followed by backcrossing to SJL for 10 generations and subsequent brother-sister mating. 9.13 TcR transgene-positive mice were further crossed with SJL $Rag^{+/-}$ mice (kindly provided by Dr. V. Kuchroo), and via selective breeding, 9.13 TCR-positive $Rag^{-/-}$ progeny (**9.13 T/R$^-$**) mice were obtained. The 9.13 $T/R^{-/-}$ will be used as donors of naïve T cells.

Hypothesis #1: p2495-specific Th17 cells are thyroidogenic in SJL mice.

Previous studies have shown that EAT and SAT are mediated by Th1 cells (Yu et al. 2002, Stafford, Rose 2000). In the ISAT model however, it was reported that IFN-γ and IL-17 mRNA were detected in thyroidal lymphocyte infiltrates of WT NOD.$H2^{h4}$ mice. Also, IL-$17^{-/-}$ NOD.$H2^{h4}$ mice were reported to have reduced ISAT symptoms (Horie et al. 2009). To examine whether Th17 cells are pathogenic in autoimmune thyroiditis, $CD4^+$ T cells from 9.13 $T/R^{-/-}$ mice will be purified from spleen and LN and will be further sorted as naïve $CD4^+$ $CD25^-$ $CD62L^{hi}$ $CD44^{lo}$ T cells. These naive cells will be cultured in the presence of anti-CD3/CD28 microbeads for 4 days under Th17 or Th1 skewing conditions. The Th17 skewing conditions would include addition of IL-23 alone (McGeachy et al. 2007) or IL-23 +IL-6 +IL1β (Lee et al. 2012, Singh et al. 2013) in the wells to generate highly pathogenic Th17 cells as well as IL-23 + IL-6 +TGF-β1

(Ghoreschi et al. 2010) to generate the classical Th17 cells. The Th1 conditions will include addition of IL-12 and anti-IL-4 Ab.

After the 4-day culture, mRNA will be purified and the expression levels of *Il-17a, Il-17f, Ifn-g, Il-21, Il-22, Il-23r, Il-4, Il-10, Tbx21* (encodes T-bet), *Rorc* (encodes RORγt), *Csf2* (encodes GM-CSF), *Foxp3* and *Gata3* will be analyzed by qPCR to confirm the differentiation towards the Th17 or Th1 lineage. Also, the presence of IL-17, GM-CSF, IL-22, IFN-γ, IL-4 and IL-10 at the protein level will be verified by intracellular cytokine staining. Once we have confirmed the *in vitro* differentiation towards the Th17 lineage, cells will be adoptively transferred i.v. to WT SJL recipient mice and EAT will be examined 2-3 weeks later. As positive control, mice will receive similarly treated T cells skewed towards the Th1 lineage i.v. If our hypothesis is correct, we expect that at least one group of *in vitro* Th17 differentiated cells will be thyroidogenic in SLJ mice. A pitfall in this approach is that Th17 may convert to Th1 cells *in vivo* due to plasticity. To examine this possibility, thyroidal T cell infiltrates will be stained, gated on $CD4^+$ $Vb4^+$ cells, a subset expected to be enriched with 9.13 T cells, and analyzed by intracellular cytokine staining for IL-17 and IFN-γ production.

In summary, our studies on the immunoregulation of autoimmune thyroiditis examined:

1) The immunopathogenicity and/or immunodominance of putative A^k-binding epitopes located close to possible cathepsin B, D and L cleavage sites in mTg. Two new immunopathogenic but cryptic Tg peptides, p2369 and p2439 were identified. This increased the number of known pathogenic Tg epitopes to 27 and we proposed possible explanations for the inability to find a dominant epitope in Tg; 2) The effect of iodide on the apoptosis of NOD.$H2^{h4}$ thyrocytes. Our data showed high susceptibility of NOD.$H2^{h4}$ vs CBA/J thyrocytes to iodide-induced apoptosis which was associated with an impaired control of oxidative stress mechanisms in the iodide-treated NOD.$H2^{h4}$ group. These results suggested that iodide-mediated apoptosis of NOD.$H2^{h4}$ thyrocytes may be the trigger of ISAT onset in this strain; 3) The effect of iodide on the spontaneous activation of thyroid-antigen specific T cells in iodide-fed animals. Activated T4p2553-specific T cells were detected in CLN of NOD.$H2^{h4}$ mice before ISAT onset as well as in intrathyroidal infiltrates. Our work identified for the first time a Tg epitope, T4p2553, as a spontaneous T cell target in ISAT providing opportunities for the construction of a TCR transgenic system in this strain; 4) The role of high salt diet in autoimmune thyroiditis development. Mice on a high salt diet did not develop significantly higher levels of the disease compared to mice on normal diet using three models of thyroiditis. These data suggested that high salt diet is not a risk factor for autoimmune thyroiditis and autoimmune diseases in general.

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Appendices

Appendix 1. Gene expression profile of apoptosis-related genes in iodide-treated NOD.H2^{h4} and CBA/J thyrocytes.

RefSeq	Symbol	Description	Fold change (+ NaI / - NaI)	
			CBA/J	NOD.H2^{h4}
NM_009594	Abl1	C-Abl oncogene 1, non-receptor tyrosine kinase	-2.07	-1.47
NM_012019	Aifm1	Apoptosis-inducing factor, mitochondrion-associated 1	1.49	1.07
NM_009652	Akt1	Thymoma viral proto-oncogene 1	-1.69	-1.70
NM_009673	Anxa5	Annexin A5	-1.18	1.23
NM_009684	Apaf1	Apoptotic peptidase activating factor 1	-1.82	-1.29
NM_007466	Api5	Apoptosis inhibitor 5	-1.38	-1.04
NM_030693	Atf5	Activating transcription factor 5	-2.26	-1.84
NM_007522	Bad	BCL2-associated agonist of cell death	1.18	1.10
NM_009736	Bag1	Bcl2-associated athanogene 1	1.18	-1.01
NM_013863	Bag3	Bcl2-associated athanogene 3	-1.11	-1.16
NM_007523	Bak1	BCL2-antagonist/killer 1	-1.33	-1.14
NM_007527	Bax	Bcl2-associated X protein	-1.28	1.03
NM_009740	Bcl10	B-cell leukemia/lymphoma 10	-1.55	-1.18
NM_009741	Bcl2	B-cell leukemia/lymphoma 2	-1.57	1.72
NM_009742	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	-1.29 [a]	1.12
NM_009743	Bcl2l1	Bcl2-like 1	-1.61	-1.20
NM_013479	Bcl2l10	Bcl2-like 10	-2.15	-1.51
NM_009754	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-1.37	1.15
NM_007537	Bcl2l2	Bcl2-like 2	-1.73	-1.38
NM_007544	Bid	BH3 interacting domain death agonist	-1.49	1.31
NM_007465	Birc2	Baculoviral IAP repeat-containing 2	-1.39	1.27
NM_007464	Birc3	Baculoviral IAP repeat-containing 3	-1.46	1.47
NM_009689	Birc5	Baculoviral IAP repeat-containing 5	1.43	1.36
NM_016787	Bnip2	BCL2/adenovirus E1B interacting protein 2	-1.27	-1.03
NM_009760	Bnip3	BCL2/adenovirus E1B interacting protein 3	-1.16	1.27
NM_009761	Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	-1.38	1.15
NM_016778	Bok	BCL2-related ovarian killer protein	-4.06	-527.93
NM_130859	Card10	Caspase recruitment domain family, member 10	-3.80	-1.07
NM_009807	Casp1	Caspase 1	-1.50	-1.16
NM_009808	Casp12	Caspase 12	-1.29	1.05
NM_009809	Casp14	Caspase 14	1.69	-2.19 [a]
NM_007610	Casp2	Caspase 2	-1.18	-1.08
NM_009810	Casp3	Caspase 3	-1.55	-1.21
NM_007609	Casp4	Caspase 4, apoptosis-related cysteine peptidase	-1.23	1.27
NM_009811	Casp6	Caspase 6	-1.20	1.33
NM_007611	Casp7	Caspase 7	-1.22	1.03
NM_009812	Casp8	Caspase 8	-1.33	1.45
NM_015733	Casp9	Caspase 9	-2.47	1.01
NM_011611	Cd40	CD40 antigen	1.12	1.99
NM_011616	Cd40lg	CD40 ligand	-3.68 [c]	-1.92 [c]

NM_011617	Cd70	CD70 antigen	-1.58 [b]	-1.18
NM_009805	Cflar	CASP8 and FADD-like apoptosis regulator	-1.68	-1.25
NM_007702	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	1.19	1.76
NM_009894	Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	1.07	1.19
NM_009950	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	-1.43	1.24
NM_010015	Dad1	Defender against cell death 1	1.31	1.41
NM_029653	Dapk1	Death associated protein kinase 1	-1.69	-1.00
NM_010044	Dffa	DNA fragmentation factor, alpha subunit	-1.74	-1.07
NM_007859	Dffb	DNA fragmentation factor, beta subunit	-2.13	1.61
NM_023232	DiAblo	Diablo homolog (Drosophila)	-1.03	1.16
NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain	-1.81	-1.08
NM_007987	Fas	Fas (TNF receptor superfamily member 6)	-1.61	1.09
NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	-1.44 [b]	-1.92 [c]
NM_007836	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	-1.57	1.09
NM_010513	Igf1r	Insulin-like growth factor I receptor	-1.74	-1.17
NM_010548	Il10	Interleukin 10	-2.67	-1.83
NM_010712	Lhx4	LIM homeobox protein 4	-3.68 [c]	-3.48
NM_010736	Ltbr	Lymphotoxin B receptor	-1.94	-1.27
NM_011949	Mapk1	Mitogen-activated protein kinase 1	-1.35	1.03
NM_008562	Mcl1	Myeloid cell leukemia sequence 1	-1.83	-1.46
NM_008670	Naip1	NLR family, apoptosis inhibitory protein 1	-17.00 [b]	-1.67
NM_010872	Naip2	NLR family, apoptosis inhibitory protein 2	1.27	1.02
NM_008689	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	-1.55	1.02
NM_080637	Nme5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	-1.43	1.79
NM_172729	Nod1	Nucleotide-binding oligomerization domain containing 1	-1.22	-1.08
NM_030152	Nol3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	1.19	1.04
NM_011130	Polb	Polymerase (DNA directed), beta	-1.16	1.02
NM_011563	Prdx2	Peroxiredoxin 2	1.08	1.20
NM_023258	Pycard	PYD and CARD domain containing	1.02	1.31
NM_009068	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	-2.11	-1.09
NM_013693	Tnf	Tumor necrosis factor	1.23	-1.18
NM_020275	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	-1.59	1.14
NM_008764	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-1.76	-1.26
NM_011609	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	-1.34	-1.24
NM_009425	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	2.36	1.22
NM_011614	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	-1.22	1.08
NM_009421	Traf1	Tnf receptor-associated factor 1	1.34	1.10
NM_009422	Traf2	Tnf receptor-associated factor 2	-1.43	1.18
NM_011632	Traf3	Tnf receptor-associated factor 3	-1.63	-1.20
NM_011640	Trp53	Transformation related protein 53	-1.26	1.12
NM_173378	Trp53bp2	Transformation related protein 53 binding protein 2	-1.51	1.04

NM_011641	Trp63	Transformation related protein 63	-1.62	1.57
NM_011642	Trp73	Transformation related protein 73	-2.10 [a]	1.06
NM_009688	Xiap	X-linked inhibitor of apoptosis	-1.62	-1.16
NM_007393	Actb	Actin, beta	-1.22	-1.36
NM_009735	B2m	Beta-2 microglobulin	1.56	1.41
NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	1.03	-1.33
NM_010368	Gusb	Glucuronidase, beta	-1.14	1.12
NM_008302	Hsp90Ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	-1.15	1.14

For certain indicated genes expression analysis was not made possible due to: (a) nonspecific amplification of the product as indicated by the melting curve analysis; (b) a high threshold cycle (30-35 cycles) in both control and test samples; and (c) an undetermined threshold cycle i.e. greater than the defined cutoff (35 cycles) in both control and test samples.

Appendix 2. Gene expression profile of oxidative stress-related genes in NaI-treated NOD.H2^{h4} and CBA/J thyrocytes.

RefSeq	Symbol	Description	Fold change (+ NaI / - NaI)	
			CBA/J	NOD.H2^{h4}
NM_009654	Alb	Albumin	-2.43 [c]	-3.31 [b]
NM_028717	Als2	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	-1.42	-1.19
NM_009676	Aox1	Aldehyde oxidase 1	-1.34	1.37
NM_007462	Apc	Adenomatosis polyposis coli	1.04	1.02
NM_009696	Apoe	Apolipoprotein E	1.80	1.34
NM_019864	Atr	Ataxia telangiectasia and rad3 related	1.09	1.24
NM_009804	Cat	Catalase	1.42	1.14
NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	2.26	1.17
NM_016892	Ccs	Copper chaperone for superoxide dismutase	1.38	-1.18
NM_007798	Ctsb	Cathepsin B	1.23	-1.27
NM_007806	Cyba	Cytochrome b-245, alpha polypeptide	1.29	1.08
NM_030206	Cygb	Cytoglobin	1.47	-2.19
NM_001039520	Dnm2	Dynamin 2	-1.01	-1.82
NM_001099297	Duox1	Dual oxidase 1	1.48	-1.17
NM_153068	Ehd2	EH-domain containing 2	1.28	-1.77
NM_007946	Epx	Eosinophil peroxidase	-1.91 [b]	-1.07 [b]
NM_007949	Ercc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	-1.11	-1.60
NM_001081221	Ercc6	Excision repair cross-complementing rodent repair deficiency, complementation group 6	1.25	-1.18
NM_007985	Fancc	Fanconi anemia, complementation group C	-1.09	-1.20
NM_018881	Fmo2	Flavin containing monooxygenase 2	2.07	-1.13
NM_010239	Fth1	Ferritin heavy chain 1	2.05	-1.02
NM_010295	Gclc	Glutamate-cysteine ligase, catalytic subunit	1.47	1.22
NM_008129	Gclm	Glutamate-cysteine ligase, modifier subunit	1.69	1.28
NM_008160	Gpx1	Glutathione peroxidase 1	1.76	-1.07
NM_030677	Gpx2	Glutathione peroxidase 2	3.14	2.55
NM_008161	Gpx3	Glutathione peroxidase 3	1.64	-1.56
NM_008162	Gpx4	Glutathione peroxidase 4	2.03	-1.04
NM_010343	Gpx5	Glutathione peroxidase 5	-1.07 [b]	2.34 [b]
NM_145451	Gpx6	Glutathione peroxidase 6	1.74 [b]	2.57 [b]
NM_024198	Gpx7	Glutathione peroxidase 7	2.64	-1.04
NM_010344	Gsr	Glutathione reductase	1.69	-1.06
NM_008180	Gss	Glutathione synthetase	1.48	-1.05
NM_029555	Gstk1	Glutathione S-transferase kappa 1	1.72	1.12
NM_013541	Gstp1	Glutathione S-transferase, pi 1	1.81	1.08
NM_010442	Hmox1	Heme oxygenase (decycling) 1	1.99	1.20

NM_010479	Hspa1a	Heat shock protein 1A	1.96	1.02
NM_010497	Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	1.86	1.05
NM_026298	Ift172	Intraflagellar transport 172 homolog (Chlamydomonas)	1.03	-1.33
NM_001009940	Il19	Interleukin 19	-1.03 [b]	1.02 [b]
NM_016971	Il22	Interleukin 22	2.16 [b]	-1.22
NM_008473	Krt1	Keratin 1	1.09 [a]	-3.64
NM_080420	Lpo	Lactoperoxidase	1.02 [a]	2.57 [b]
NM_013593	Mb	Myoglobin	2.01	-1.49
NM_010824	Mpo	Myeloperoxidase	3.20	-1.40
NM_010876	Ncf1	Neutrophil cytosolic factor 1	1.28	-1.77
NM_010877	Ncf2	Neutrophil cytosolic factor 2	2.96	-1.03
NM_022414	Ngb	Neuroglobin	1.38	-1.94
NM_010927	Nos2	Nitric oxide synthase 2, inducible	-1.05 [a]	-5.40 [a]
NM_172203	Nox1	NADPH oxidase 1	2.30 [b]	1.26 [b]
NM_015760	Nox4	NADPH oxidase 4	1.92	-1.54
NM_172204	Noxa1	NADPH oxidase activator 1	4.11 [a]	2.97 [a]
NM_027988	Noxo1	NADPH oxidase organizer 1	1.42	2.09
NM_008706	Nqo1	NAD(P)H dehydrogenase, quinone 1	2.89	2.37
NM_020569	Park7	Parkinson disease (autosomal recessive, early onset) 7	2.49	-1.03
NM_011034	Prdx1	Peroxiredoxin 1	2.97	1.12
NM_011563	Prdx2	Peroxiredoxin 2	2.43	-1.03
NM_007452	Prdx3	Peroxiredoxin 3	1.82	1.02
NM_016764	Prdx4	Peroxiredoxin 4	1.91	-1.16
NM_012021	Prdx5	Peroxiredoxin 5	2.27	-1.03
NM_007453	Prdx6	Peroxiredoxin 6	1.91	-1.17
NM_011170	Prnp	Prion protein	1.48	-1.21
NM_011186	Psmb5	Proteasome (prosome, macropain) subunit, beta type 5	2.18	1.04
NM_008969	Ptgs1	Prostaglandin-endoperoxide synthase 1	1.42	-1.31
NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2	1.37	-1.30
NM_009020	Rag2	Recombination activating gene 2	1.08	1.91
NM_058214	Recql4	RecQ protein-like 4	1.53	-1.55
NM_009127	Scd1	Stearoyl-Coenzyme A desaturase 1	1.92	-1.12
NM_173052	Serpinb1b	Serine (or cysteine) peptidase inhibitor, clade B, member 1b	2.87	1.08
NM_134086	Slc38a1	Solute carrier family 38, member 1	1.19	-1.39
NM_011434	Sod1	Superoxide dismutase 1, soluble	3.57	1.02
NM_013671	Sod2	Superoxide dismutase 2, mitochondrial	1.38	-1.35
NM_011435	Sod3	Superoxide dismutase 3, extracellular	1.20	-1.56
NM_011018	Sqstm1	Sequestosome 1	1.18	1.20
NM_029688	Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	1.22	1.04
NM_009417	Tpo	Thyroid peroxidase	5.77	1.65
NM_011660	Txn1	Thioredoxin 1	2.13	1.40
NM_023719	Txnip	Thioredoxin interacting protein	2.62	1.40
NM_015762	Txnrd1	Thioredoxin reductase 1	1.63	-1.26
NM_013711	Txnrd2	Thioredoxin reductase 2	1.93	-1.09
NM_153162	Txnrd3	Thioredoxin reductase 3	1.41	-1.21
NM_011671	Ucp2	Uncoupling protein 2 (mitochondrial, proton carrier)	2.01	-1.24

NM_009464	Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	2.15	-1.67
NM_011701	Vim	Vimentin	1.88	-1.21
NM_011728	Xpa	Xeroderma pigmentosum, complementation group A	1.51	-1.00
NM_007393	Actb	Actin, beta	-1.10	-1.36
NM_009735	B2m	Beta-2 microglobulin	-1.19	1.31
NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	1.27	-1.16
NM_010368	Gusb	Glucuronidase, beta	1.03	1.21
NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1	1.00	-1.00

For certain indicated genes expression analysis was not made possible due to: (a) nonspecific amplification of the product as indicated by the melting curve analysis; (b) a high threshold cycle (30-35 cycles) in both control and test samples; and (c) an undetermined threshold cycle i.e. greater than the defined cutoff (35 cycles) in both control and test samples.