IMMUNE RESPONSES TO THYROGLOBULIN PEPTIDES IN ANIMAL MODELS OF EXPERIMENTAL AUTOIMMUNE THYROIDITIS

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by

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LIST OF ABBREVIATIONS

AA	Adjuvant Arthritis
a.a.	Amino Acid
Ab	Antibody
AITD	Autoimmune Thyroid Disease
APC	Antigen Presenting Cell
AutoAb	Autoantibody
Bhsp65	Bacterial 65-kDa Heat Shock Protein
BM	Bone Marrow
BM-DC	Bone Marrow-Derived Dendritic Cells
bTg	Bovine Thyroglobulin
BUF	Buffalo
CFA	Complete Freund's Adjuvant
CLT	Chronic Lymphocytic Thyroiditis
conA	Concavalin A
CTLL	Cytotoxic T Lymphocyte Line
CNS	Central Nervous System
DIT	Diiodotyrosine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsufoxide
EAE	Experimental Autoimmune Encephalomyelitis

EAT	Experimental Autoimmune Thyroiditis
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplamic Reticulum
FBS	Fetal Bovine Serum
GAD	Glutamic Acid Decarboxylase
GD	Graves' Disease
GM-CSF	Granulocyte, Macrophage, Colony Stimulating Factor
H&E	Haematoxylin and Eosin
hsp65	Heat Shock Protein
HAT	Hypoxanthine-Aminopterin-Thymidine
HI-Tg	Highly Iodinated Thyroglobulin
HT	Hashimoto's Thyroiditis
hTg	Human Thyroglobulin
I	Iodide
I_2	Iodine
IA-SAT	Iodine-Accelerated Spontaneous Autoimmune Thyroiditis
IDDM	Insulin Dependent Diabetes Mellitus
IFA	Incomplete Freund's Adjuvant
I.I.	Infiltration Index
IL-2	Interleukin-2
i.p.	Intraperitoneal
i.v.	Intravenous
LNC	Lymph Node Cells

LPS	Lipopolysaccaride
mAbs	Monoclonal Antibodies
2-ME	2-Mercaptoethanol
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MIT	Mono-iodotyrosine
MOG	Mylein Oligodendrocyte
MS	Multiple Sclerosis
mTg	Mouse Thyroglobulin
Na^+	Sodium
NaI	Sodium Iodide
Na^+/K^+-ATPase	Sodium Potassium -ATPase
NIS	Sodium Iodide Symporter
NOD	Non-obese Diabetic
O.D.	Optical Density
OS	Obese Strain
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PBS-T	PBS with Tween-20
PLP	Proteolipid Protein
pTg	Porcine Thyroglobulin
PTX	Pertussis Toxin
RA	Rheumatoid Arthritis

RBC	Red Blood Cell
SAT	Spontaneous Autoimmune Thyroiditis
s.c.	Subcutaneous
S.I.	Stimulation index
T_3	Triiodothyronine
T_4	Thyroxine
TAC	Tetrameric Antibody Complexes
TDLN	Thyroid draining lymph nodes
Tg	Thyroglobulin
T_H1	T Helper Type 1 Cells
TPO	Thyroid Peroxidase
TSH	Thyroid Stimulating Hormone (Thyrotrophin)
T_{regs}	Regulatory T Cells

CHAPTER 1

INTRODUCTION

1.1 The Thyroid

The thyroid is a bilobed organ located in the neck and is the largest organ that functions exclusively as an endocrine gland (Capen, 2000). The two lobes of the thyroid are connected by the isthmus which lies over the trachea. The basic structure of the thyroid consists of many, different sized, follicles that are filled with colloid produced by follicular cells (Capen, 2000). These cells contain rough endoplasmic reticulum (ER) and a large Golgi apparatus in the cytoplasm, structures important for the synthesis of the thyroid proteins thyroglobulin (Tg) and thyroid peroxidase (TPO) (Capen, 2000). The main function of the thyroid is to produce and secrete the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) (Dunn & Dunn, 2001), which are important for regulating metabolic processes in the body. These processes include growth, development, oxygen consumption, and protein, lipid, carbohydrate and vitamin metabolism (Yen, 2005).

1.1.1 Thyroid Stimulating Hormone (TSH) and thyroid regulation

The process of hormone synthesis and secretion in the thyroid is regulated by thyroid stimulating hormone (TSH) or thyrotrophin, a glycoprotein produced by the pituitary gland. This protein binds to its TSH receptor (TSHR), which is found mainly on the surface of thyroid follicular cells (Santisteban, 2005). Binding of TSH to its TSHR increases the endocytosis of thyroglobulin (Tg), leading to increased synthesis and

secretion of thyroid hormone, as well as the differentiation and proliferation of the thyroid (Santisteban, 2005). Further, the production of TSH is under the control of thyrotrophin-releasing hormone (TRH) which is secreted by the hypothalamus and acts on the anterior pituitary gland. A negative feedback loop is the key to the regulation of hormone synthesis and secretion.

1.2 Hashimoto's thyroiditis (HT)

Autoimmune thyroid disease (AITD) specifically targets the thyroid and is characterized by lymphocytic infiltration of the gland as well as the production of thyroid autoantibodies (autoAb) in the serum (Weetman, 2004). AITD is not a disease itself, but a collection of diseases. Two clinically significant AITD diseases are Graves' disease (GD) which affects ~ 1% of women (Weetman, 2000) and Hashimoto's thyroiditis (HT) which affects 1-2% of women (Pearce *et al.*, 2003). HT or chronic lymphocytic thyroiditis (CLT) is the most common AITD in humans (Pearce *et al.*, 2003).

HT or struma lymphomatosa was first described by the Japanese surgeon, Hakaru Hashimoto in 1912 (Hashimoto, 1912; Aozasa, 1990). This disease affects mainly middle aged women at an 8:1 to 9:1 female to male ratio (Pearce *et al.*, 2003). In HT, once thyroid antigen-specific helper T cells become activated the anti-thyroid immune response begins. The activated helper T cells cause: 1) B cells to secrete autoAb against thyroid autoantigens and 2) cytotoxic T cells to become activated to home to, infiltrate, and destroy the thyroid gland. However, the mechanisms involved in the initial activation

of these helper T cells are still unknown (Pearce *et al.*, 2003). It has been reported that thyrocytes from patients with HT express the Fas gene, whereas thyrocytes from patients without HT do not (Pearce *et al.*, 2003). Furthermore, $CD4^+$ killer T cells express Fas Ligand (FasL) which is able to bind to thyrocytes from patients with HT and cause FasL-medicated apoptosis (Pearce *et al.*, 2003). The gradual destruction of the thyroid gland by lymphocytic infiltration, the production of autoAb in the serum which are specific for the thyroid autoantigens, Tg (Roitt I.M *et al.*, 1956) and TPO (Weetman & McGregor, 1994), and formation of goiter (Pearce *et al.*, 2003) are all characteristics of HT. It has been shown that susceptibility to HT is under the control of both genetic (human leukocyte antigen (HLA-DR) alleles, cytotoxic T lymphocyte antigen (CTLA)-4 polymorphisms, and thyroid specific genes, like Tg) and environmental (iodine intake) factors (Ban & Tomer, 2005; Weetman, 2004). Tg has also been found to play a role in the susceptibility of thyroiditis. Ban and Tomer studied the Tg gene which is located on chromosome 8q24 and found that there were susceptibility genes linking to AITD (Ban & Tomer, 2005). From this gene they identified two new Tg microsatellites in intron 10 (Tgms1) and intron 27 (Tgms2) which through familial studies showed that only Tgms2 was linked and associated with AITD, making it a an important AITD susceptibility gene (Ban & Tomer, 2005). It has also been documented that HT is the leading cause of hypothyroidism and goiter in humans who live in countries where dietary iodine intake is sufficient (Pearce *et al.*, 2003).

1.2.1 Hypothyroidism in HT

The development of hypothyroidism in patients with HT is a consequence of depleted circulating hormone levels. These hormones become depleted as a result of decreasing production and secretion of T_4 and T_3 by the thyroid (Braverman and Utiger, 2005). This may occur due to destruction of the thyroid gland by mononuclear cell infiltration. As the function of the thyroid gland begins to fail, a person with HT will develop subclinical hypothyroidism, showing normal hormone levels and elevated TSH concentrations in their serum. As the disease progresses and thyroid function fails, TSH concentrations become elevated (normal range 0.4 – 4.0 mIU/L; Hurd, 2007) and T_4 concentrations decrease in the serum. Normal T_4 levels are between 5-13.5 µg/dL (Evans, 2005). This stage of hypothyroidism is called overt hypothyroidism. The T_3 levels (normal range 1.2-3.4 nmol/L) in the serum usually drop sometime after the T_4 levels, however, once the T_3 level drops, a patient will experience the classical signs of HT (Pearce *et al.*, 2003) such as goiter, constant fatigue, poor memory, constipation, etc. (Ginsberg *et al.*, 2007).

1.3 Experimental autoimmune thyroiditis (EAT)

Experimental autoimmune thyroiditis (EAT) is the animal model of HT. There are many animal models which have been used to study EAT, such as the rabbit (Rose & Witebsky, 1956), the mouse (Rose *et al.*, 1971), the guinea-pig, the dog (Terplan *et al.*, 1960), the rat (Jones and Roitt, 1961), the chicken (Jankovic, 1965), and the rhesus monkey (Rose *et*

al., 1966). These models are Tg-induced EAT models, but they are considered ideal in the study of HT because they show similarities in development of the disease. This is evident in the destruction of the thyroid gland by mononuclear cell infiltration and the production of autoantibodies (autoAb) to Tg in the serum.

1.3.1 Thyroglobulin-induced EAT in the mouse

In order to study EAT in animals which do not develop the disease spontaneously, disease must be induced either directly or indirectly. Direct induction of EAT requires animals to be challenged with a thyroid antigen in adjuvant. This method has been used since 1956, when Rose and Witebsky first immunized rabbits subcutaneously (s.c.) with homologous thyroid tissue in complete Freund's adjuvant (CFA) (Rose and Witebsky, 1956). They discovered that rabbits who received these injections showed lymphocytic infiltration in their thyroid glands and produced thyroid autoAb in their serum. Since 1956, many methods have been used to induce EAT in the mouse. They include challenge with mouse thyroglobulin (mTg) in different types of adjuvants such as lipopolysaccharide (LPS) and CFA (Esquivel *et al.*, 1977), mTg conjugated to monoclonal antibodies instead of adjuvants (Balasa & Carayanniotis, 1993), and repeated injections of large amounts of Tg in small doses in the absence of adjuvant (Elrehewy *et al.*, 1981). All these methods were successful in eliciting IgG production to mTg. However, not all methods caused mononuclear cell infiltration in the thyroid gland (Balasa & Carayanniotis, 1993). The most commonly used technique for inducing EAT in mice is through s.c. injection with

homologous Tg in CFA followed by a booster injection of Tg in incomplete Freund's adjuvant (IFA) 2-3 weeks later (Charreire, 1989; Weetman & McGregor, 1984; Weetman & McGregor, 1994). This method successfully leads to the induction of EAT whereby antibodies (Ab) to mTg are produced in the serum and extensive infiltration is detected in the thyroid gland.

EAT can also be indirectly induced by adoptive transfer of lymph node or spleen cells from animals immunized with Tg in CFA into naïve syngeneic recipients. EAT appears 2-3 weeks post-transfer and is evident by mononuclear cell infiltration in the gland and the production of Ab to Tg in the serum (Braley-Mullen *et al.,* 1985). Another method used to indirectly induce EAT is thymectomy with repeated sub-lethal irradiation. This procedure may be performed in animals immediately after birth or later in life. One study showed that 60% of the thymectomized Wistar rats which received whole body irradiation developed EAT spontaneously, 60 days after the last irradiation compared to 0% of the untreated control rats (Penhale *et al.,* 1973). It has been hypothesized that this method selectively depletes T cells responsible for suppressing EAT (Penhale *et al.*, 1976). With the depletion of this population, there is a loss of tolerance to self-thyroid antigens and the development of EAT (Penhale *et al.*, 1976). A second study showed that the putative suppressor cells were of thymic origin and that reconstituting thymectomized rats with normal lymphoid or spleen cells during the development of disease could suppress EAT (Penhale *et al.,* 1976). Even under optimal conditions, only certain strains of mice are susceptible to Tg-induced EAT. This susceptibility is under the genetic

control of the major histocompatibility (MHC) gene, *H2*. A study by Vladutiu and Rose showed that there are good and poor responder mouse strains in Tg-induced EAT (Vladutiu & Rose, 1971). They immunized 33 inbred strains of mice representing 11 *H2* types, twice (7-day intervals) with thyroid extract emulsified in CFA. They discovered that there were differences in immune responses in mice with different *H2* haplotypes (Vladutiu & Rose, 1971). Mice which are $H2^s$ or $H2^k$ are considered good responder strains because they produce high titers of Ab to mTg and show extensive mononuclear cell infiltration in their thyroids. On the other hand, mice which are $H2^d$ and $H2^b$ are poor responder strains because they produce very low levels of Ab to mTg in their serum and show little if any infiltration in their thyroid glands after immunization with thyroid extracts in CFA (Vladutiu & Rose, 1971).

1.3.2 Spontaneous autoimmune thyroiditis (SAT)

Spontaneous autoimmune thyroiditis (SAT) can develop in many animal models, such as Obese strain (OS) chickens (Witebsky *et al.*, 1969), Buffalo (BUF) rats (Hajdu & Rona, 1969), and non obese diabetic NOD.$H2^{h4}$ mice (Rasooly *et al.*, 1996). In NOD.$H2^{h4}$ mice, SAT is characterized by the destruction of the thyroid gland by mononuclear cell infiltration and the production of the Tg autoantibodies, IgG1 and IgG2b (Braley-Mullen *et al.*, 1999; Rasooly *et al.*, 1996) which is similar to HT. The cells mainly involved in the destruction of the thyroid gland are comprised of $CD4^+$ and $CD8^+$ T cells, and $B220^+$ cells (Yu *et al.*, 2006). It has been documented that the production of IgG1 and IgG2b Ab

correlate with SAT severity scores (Braley-Mullen *et al.,* 1999). However, it has also been documented that the production of IgG2b only, correlated with SAT severity (Rasooly *et al.*, 1996).

1.3.2.1 SAT in NOD.H2^{h4} mice

The NOD.H2^{h4} mouse strain was developed during a study which tested the role of I-E MHC expression on the development of insulitis and diabetes in mice with the NOD background (Podolin *et al.*, 1993). NOD.H2^{h4} mice were derived by crossing NOD(H2^{g7}) mice (which spontaneously develop type I diabetes (Kikutani & Makino, 1992) and have a low incidence of thyroiditis (Damotte *et al.,* 1997), as seen in histological examinations of NOD pancreata and thyroids) with B10.A(4R)(H2^{h4}) mice (which express the A^k allele making them susceptible to thyroiditis (Vladutiu & Rose, 1971)). The heterozygous progeny from the first generation, expressing the A^k gene were repeatedly backcrossed with NOD mice until the sixth generation, in which brother-sister matings (H2$^{h4/h4}$) occurred (Podolin *et al.,* 1993). The resulting NOD.H2^{h4} mice produced are E^- and express A^k, K^k, and D^b on the NOD background (Podolin *et al.*, 1993). These mice develop SAT (Rasooly *et al.*, 1996), some insulitis, but no diabetes (Podolin *et al.,* 1993; Weatherall *et al.,* 1992) compared to their parental strain.

According to Braley-Mullen *et al.,* untreated NOD.H2^{h4} mice do not develop SAT before the age of 6 months (Braley-Mullen *et al.,* 1999). However, studies have shown that supplementing 0.05% sodium iodide (0.05% NaI) in the drinking water of 6-8 week old

NOD.H2^{h4} mice, for 8 weeks, accelerates the onset and increases the severity of SAT in the thyroid, as well as the production of Tg Ab (mostly IgG2b and some IgG1) in the serum (Braley-Mullen *et al.*, 1999; Rasooly *et al.*, 1996). Also, Rasooly *et al.* showed that the addition of NaI in the drinking water of NOD.H2^{h4} mice had no affect on serum thyroxine levels and antibodies to TPO could not be detected in both iodine-treated and control mice (Rasooly *et al.*, 1996).

Studies have shown that CD4$^+$ and CD8$^+$ T cells are essential for SAT development (Braley-Mullen *et al.*, 1999; Hutchings *et al.*, 1999). Depletion of CD4$^+$ or CD8$^+$ T cells in NOD.H2^{h4} mice before SAT development minimized or prevented SAT and decreased anti-mTg autoAb in the serum. However, in mice which had already developed SAT, they found that only CD4$^+$ T cell depletion could minimize or prevent SAT development. Also, they found that CD4$^+$ T cell depletion only slightly reduced anti-mTg autoAb levels in the serum compared to control mice who had their CD4$^+$ T cells intact (Braley-Mullen *et al.*, 1999). The depletion of both CD4$^+$ and CD8$^+$ T cells in NOD.H2^{h4} mice showed similar results as those only having CD4$^+$ T cells depleted. Therefore, CD4$^+$ T cells are essential in the initial development and maintenance of SAT and CD8$^+$ T cells are only essential in the initial development of SAT in NOD.H2^{h4} mice (Braley-Mullen *et al.*, 1999).

The kinetics of SAT in NOD.H2^{h4} mice has also been studied. Braley-Mullen *et al.* reported that mice given 0.05% NaI water for 2 weeks showed lesions in their thyroids (Braley-Mullen *et al.*, 1999). Also, NOD.H2^{h4} mice on NaI water for 8 weeks showed

maximum SAT severity in their glands with severity of disease persisting for an additional 15 weeks (23 weeks on NaI water). Braley-Mullen *et al.* noticed that males appeared to have more severe lesion development than females. However, this was not statistically significant (Braley-Mullen *et al.*, 1999) and this was confirmed by another study (Rasooly *et al.,* 1996). The cells found infiltrating the thyroids of these mice were mainly $CD4^+$ T cells, then B cells, and then $CD8^+$ T cells (Yu *et al.*, 2001). It is important to note that SAT development also requires the presence of B cells early in life in NOD.$H2^{h4}$ mice. Braley-Mullen *et al.* reported that the absence of B cells in NOD.$H2^{h4}$ mice leads to minimal or no SAT development (Braley-Mullen & Yu, 2000). This is because B cells are required early in life when T cells are maturing. Reconstituting B cell-depleted NOD.$H2^{h4}$ mice with B cells only elevated antibody levels but had no effect on SAT development. However, if B cell-deficient NOD.$H2^{h4}$ mice were irradiated and then given bone marrow from B cell-deficient mice and B cells from normal mice, thyroiditis occurred. Therefore, T cells from B cell-deficient NOD.$H2^{h4}$ mice are capable of destroying the thyroid if they mature in the presence of B cells (Braley-Mullen & Yu, 2000). The lack of SAT development in B cell-deficient NOD.$H2^{h4}$ mice may be due to the lack of B cells activating autoreactive T cells. An experiment conducted by Yu *et al.* reported that regulatory T cells (T_{regs}) may play a role in the resistance to SAT development in B cell-deficient mice (Yu *et al.*, 2006). They reported that B cell-deficient mice have similar levels of T_{regs} in their spleens and peripheral blood as wildtype (NOD.$H2^{h4}$) mice. However, B cell-deficient mice do not develop SAT.

Therefore, this lack of SAT development is not due to elevated T_{regs} levels in B cell-deficient mice. B cell-deficient mice without T_{regs} developed SAT, revealing that the presence of T_{regs} has some role in SAT resistance in B cell-deficient mice. Maybe T_{regs} are important in inhibiting the activation of autoreactive T cells required for SAT development in these mice (Yu *et al.*, 2006).

1.4 Thyroglobulin

1.4.1 Biosynthesis of Tg

Tg, the major autoantigen in autoimmune thyroiditis, is a large homodimeric glycoprotein weighing ~660 kDa (Dunn & Dunn, 2000). Tg is produced, stored, and secreted from the follicular lumen of the thyroid follicles. The production of Tg is physiologically controlled by the pituitary glycoprotein TSH (Dunn & Dunn, 2000) and its main purpose is to provide a matrix for the synthesis of the thyroid hormones T_3 and T_4. Before Tg can aid in the synthesis of these hormones, a series of posttranslational modifications have to occur. Firstly, the unfolded immature Tg molecule must enter the endoplasmic reticulum (ER) where it is folded into a stable dimer. This process is facilitated by enzymes (protein disulfide isomerase and peptydylprolyl isomerase) and molecular chaperones (calnexin, BiP, GRP94, and Erp72) (Dunn & Dunn, 2000). The molecular chaperones also play a role in preventing improperly folded or premature Tg from leaving the ER. Once Tg is properly folded, it is allowed to migrate from the ER to the Golgi complex where it completes the processing of its carbohydrate units through the addition of sugars,

sulfates, and phosphates. The mature Tg molecule is now ready to be transported from the Golgi to the apical cell surface in apical vesicles. Here at the apical cell surface, the mature Tg molecule is iodinated (Dunn & Dunn, 2000). To date, the complete sequences of human thyroglobulin (hTg) (Malthiery & Lissitzky, 1987), bovine thyroglobulin (bTg) (Mercken *et al.*, 1985) and mTg (Caturegli *et al.*, 1997) have been documented. The mature sequences for each are as follows: hTg contains 2748 amino acids (a.a.), bTg contains 2750 a.a and mTg contains 2748 a.a (Caturegli *et al.*, 1997; Malthiery & Lissitzky, 1987; Mercken *et al.*, 1985). It has also been shown that mTg is 71.8 % and 73.5 % homologous to hTg and bTg, respectively

1.4.2 Iodine in Tg

In vertebrates, the thyroid is the only tissue in the body capable of concentrating iodide (I^-). Tg incorporates and stores available iodine (I_2) in the form of iodotyrosyls, such as mono-iodotyrosine (MIT) and di-iodotyrosine (DIT). Intramolecular coupling of two iodotyrosyls, either two DIT or one MIT and one DIT, form the thyroid hormones T_4 and T_3, respectively (Dunn, 1995). There are four major hormonogenic sites in each monomer of Tg (Dunn & Dunn, 2000). In order for Tg to become iodinated, both mature Tg and reactive I_2 must be present on the apical cell surface of the thyrocyte. The formation of reactive I_2 is formed by the uptake of I^- from the circulation by the sodium iodide (Na^+/I^-) symporter (NIS), a plasma membrane protein (Levy *et al.*, 1997). The NIS is present on the basolateral membrane of the thyrocyte and aids in the transport of I^-

across the thyroidal cell membrane along an electrochemical gradient to the apical cell surface. This process is made possible by the Na^+ ion gradient which is the driving force for I^- uptake and is maintained by the sodium/potassium (Na^+/K^+)-ATPase (Capen, 2000). The inward flow of Na^+ ions into the thyrocyte simultaneously allows the inward flow of I^- ions into the thyrocyte (Levy *et al.*, 1997). Once I^- is transported to the follicular lumen, enzymes such as TPO and hydrogen peroxide, oxidize I^- to reactive I_2 which can now bind to tyrosyl residues within Tg (Levy *et al.*, 1997). Once Tg becomes iodinated, the thyroid hormones, mediated by TPO, can be formed (Dunn & Dunn, 2001). In order for T_3 and T_4 to be released into the circulation, they need to be cleaved from iodinated Tg. This is accomplished through the endocytosis of Tg in the form of colloid droplets. These droplets then fuse with lysosomes containing proteolytic enzymes which hydrolyze Tg and remove T_3 and T_4 from the Tg backbone (Santisteban, 2005).

1.4.3 Epitope mapping of Tg

Numerous methods have been used to screen Tg for antigenic T-cell determinants. Two of these methods have used T cell hybridoma clones specific for mTg and computerized algorithms. The first T cell epitope discovered was a 9mer Tg peptide (2551-59) which contained T4 at position 2553. The 40 mer hTg peptide (1672-1711) was found as a result of an experiment using a cytotoxic T cell hybridoma and tryptic fragments from porcine thyroglobulin (pTg) (Texier *et al.*, 1992). Synthesis of a 40-amino acid peptide

similar to a portion of hTg was tested and shown to induce lymphocytic infiltration in the thyroid glands of CBA/J mice (Texier *et al.*, 1992).

Computerized algorithms designed to predict T cell epitopes in a protein sequence (Altuvia *et al*, 1994; Margalit *et al.*, 1987; Rothbard & Taylor, 1988) have been used in our laboratory to search for A^k-restricted, EAT-causing, T cell epitopes in Tg (Carayanniotis, *et al.*, 1994; Chronopoulou & Carayanniotis, 1992; Rao *et al.*, 1994; Rao & Carayanniotis, 1997; Verginis *et al.*, 2002). Many mTg epitopes have been identified because they contain a.a. sequence motifs indicating putative binding A^k, E^k and A^s molecules. Extensive use has been made of an algorithm developed by Altuvia *et al.* that searches for A^k and E^k-binding motifs based on size, hydrophobicity, etc. important for peptide binding to A^k and E^k molecules (Altuvia *et al.*, 1994). To date, the use of computerized algorithms and hybridomas has led to the discovery of 23 Tg peptides (Carayanniotis, 2007), four of which contain hormonogenic sites (Carayanniotis, 2003), four which contain iodotyrosyl residues (Li & Carayanniotis, 2006), and 15 which do not contain iodine (Carayanniotis, 2007). Using the algorithm described by Altuvia et al., Li & Carayanniotis (2006) scanned the complete murine Tg sequence for the presence of A^k-binding motifs flanked by tyrosine residues to determine if iodotyrosyls alter the immunogenicity of Tg peptides (Li & Carayanniotis, 2006). A total of 20 sites were discovered and 13 Tg peptides and their iodinated analogues were synthesized. CBA/J mice were challenged with each Tg peptide and nine days later draining lymph nodes were collected and the cells were cultured in the presence of their respective peptides.

The results showed that the addition of iodotyrosyl residues had a variety of effects on the immunogenicity of the 13 Tg peptides. For seven of the 13 Tg peptides tested, the iodination status had no effect on the immunogenicity. However, for the Tg peptides p179, p2540, & p2529 which were immunogenic in their non-iodinated form there were variable effects in their iodinated form. They found that the addition of iodine to each of these Tg peptide sequences caused an increase, decrease, or did not alter the immunogenic profile of these Tg peptides, respectively. Further, they found that for the Tg peptides p117, p304, & p1931 which were non-immunogenic in non-iodinated form, the addition of iodotyrosyl residues increased the immunogenicity of these Tg peptides (Li & Carayanniotis, 2006).

1.4.4. Dominance and crypticity

To date, none of the Tg peptides tested have been found to be immunodominant. An epitope is considered dominant when it can be detected by T cells following processing of intact Ag *in* vivo and *in vitro* (Sercarz *et al.*, 1993). Although immunodominant epitopes have not been discovered in Tg, seven Tg peptides have been found to be subdominant (Jiang *et al.*, 2007). An epitope is subdominant when peptide-primed T cells irregularly respond to intact antigen (Ag) *in vivo* and/or *in vitro*. It is also important to note that in order to detect a subdominant epitope, the molar concentrations of intact Ag have to be much higher than those required by free peptide (Jiang *et al.*, 2007). To determine if an epitope is dominant or cryptic, proliferation assays are conducted after disease is induced

by a protein Ag (Moudgil & Sercarz, 2005). A dominant epitope is more readily available to, and has a higher affinity for binding to, MHC molecules (Sercarz, 2002) compared to cryptic epitopes. There are three reasons why some determinants are cryptic. One reason is processing; improper processing of a molecule can render a previously cryptic epitope dominant (Moudgil & Sercarz, 2005). Another reason is competition. Competition can lead to 1) the generation of cryptic epitopes by reducing the number of MHC class II binding sites and/or 2) multiple MHC molecules may bind to different determinants on the same molecule in which the dominant epitope will be presented (Moudgil & Sercarz, 2005). A final reason for the crypticity of an epitope is hindrance; T cell receptor (TCR) access to the MHC-peptide complex can be blocked by flanking amino acid residues inhibiting the binding of the TCR to the MHC-peptide complex. This phenomenon prevents the epitope from being recognized by the T cell and therefore renders the epitope cryptic (Moudgil & Sercarz, 2005).

1.5 T cell epitope spreading

There are many factors that contribute to the development of autoimmunity and one which has recently been studied is the phenomenon of epitope spreading. This process occurs when the immune response (i.e. a T cell response) to autoantigens become more diverse as the disease progresses; and can occur at the T and/or B cell level (Vanderlugt & Miller, 2002). T cell epitope spreading is the changing in specificity of the immune response to T cell epitopes. The phenomenon of T cell epitope spreading revealed that the

immune response is dynamic and that T cells which recognize dominant epitopes, over time, can recognize formerly cryptic epitopes during the course of disease (Lehmann *et al.*, 1992). Since then, many studies on epitope spreading have been conducted revealing that there are two types of epitope spreading, intra- and intermolecular epitope spreading (Vanderlugt & Miller, 2002). Intramolecular epitope spreading has been defined as the spreading of an immune response from one epitope to another epitope on the same molecule or protein; and intermolecular epitope spreading is the change in specificity of an immune response from one molecule to another molecule.

Epitope spreading can be initiated by chronic tissue damage through the activation and recruitment of autoreactive lymphocytes (Vanderlugt & Miller, 2002) or by the induction of chronic tissue pathology by viruses (Vanderlugt & Miller, 1996). Therefore, a primary response has to occur whereby the recognition of a primary epitope causes the activation and differentiation of autoreactive T helper type 1 (T_H1) cells. These cells migrate to the infected tissue, bind to APC, become activated and release cytokines, which cause tissue damage. Autoantigen complexed with autoantibodies is then processed differently and presented to APC which activate and differentiate a second wave of T_H1 cells (Vanderlugt & Miller, 2002; Dai *et al.*, 2005). These cells can now reenter the infected area and cause more tissue damage due to the recognition of other previously cryptic epitopes (epitope spreading) (Vanderlugt & Miller, 2002).

1.5.1 T cell epitope spreading in experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis (AA)

The study of T cell epitope spreading is a relatively new concept. It was first considered in 1992 by Lehmann *et al.* in their study on experimental allergic encephalomyelitis (EAE), the mouse model of multiple sclerosis (MS) (Lehmann *et al.*, 1992). EAE is caused by the autoreactivity of $CD4^+$ T_H1 cells to a single dominant myelin peptide determinant (Tuohy *et al.*, 1999), either the CNS protein MBP, PLP or myelin oligodendrocyte glycoprotein (MOG) (Vanderlugt & Miller, 2002). Studies of EAE have shown that during the course of chronic disease there is spreading of the T cell response from a single dominant peptide, to formerly cryptic peptides. Lehmann *et al.*, first studied T cell epitope spreading in EAE by immunizing (SJL x B10.PL)F1 mice with MBP and pertussis toxin (PTX, a requirement for the induction of EAE because it disrupts the blood brain barrier) (Lehmann *et al.*, 1992). Results showed that there was evidence of T cell epitope spreading from the dominant MBP:Ac1-11 peptide, to cryptic MBP peptides, 35-47, 81-100, and 121-140, i.e. the specificity of the T cell immune response was constantly changing (Lehmann *et al.*, 1992). The authors claimed that epitope spreading could be the reason that there is chronic disease, because the change in specificity for different epitopes in MBP by the T cells may lead to chronic tissue damage and the progression of EAE. Also, they noted that due to the constant generation of new epitopes,

it may be difficult to treat EAE using peptide-specific therapies (Lehmann *et al.*, 1993; Vanderlugt & Miller, 1996; Vanderlugt & Miller, 2002).

In 1997, Moudgil *et al.* tested T cell epitope spreading in the rat model of AA (Moudgil *et al.*, 1997). They induced disease in Lewis rats by s.c. injection of *M. tuberculosis* H37Ra in IFA or mineral oil in their hind footpad. Lymph node cells (LNC) were tested for T cell reactivity against a panel of Bhsp65 peptides, during both the early and late phases of disease. They found that during the early phase (6-9 days), there were T cell responses detected against 13 Bhsp65 peptides which were either dominant or subdominant. During the late phase (8-10 weeks), they found that there were T cell responses to 16 peptides, with only 2 of the 16 peptides being recognized from the early phase.

In this study we intended to investigate whether T cell epitope spreading occurs in EAT, a $CD4^+$ T cell mediated disease. For this purpose we used several "cryptic" Tg peptides as markers to examine if the autoimmune response spreads toward them during the course of disease; in order to determine if cryptic T cell determinants are generated after the challenge of Tg in CFA in CBA/J mice.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Female CBA/J mice were purchased from Jackson Laboratories (Bar Harbour, ME, USA) and used at 7-8 weeks of age. Two breeding pairs of NOD.H2^{h4} mice were a kind gift from Dr. H. Braley-Mullen. A NOD.H2^{h4} colony was established from these breeding pairs in our animal care facility. All experimental procedures were approved by the Animal Care Committee at Memorial University of Newfoundland.

2.2 Antigen

2.2.1 Tg purification

One hundred mouse thyroid glands were placed in a 16 x 100 mm culture tube (Fisher-Scientific, Ottawa, ON, CA) on ice in 5 ml of phosphate buffer saline (PBS) containing protease inhibitors (leupeptin 10^{-6} mol/L, pepstatin A 10^{-5} mol/L, and PMSF 10^{-3} mol/L ; Sigma-Aldrich, St. Louis, MO, USA). Thyroids were homogenized in short bursts using a PCR tissue homogenizing kit (OMNI International, Marietta, GA, USA) and divided into 5 Eppendorf tubes each containing 1 ml of suspension. The tubes were spun three times at 14,000 rpm for 10 minutes and supernatants were removed and placed over a Sepharose CL-4B column (Amersham Biosciences, Bale d'Urfe, Quebec, CA). PBS with protease inhibitors was then run through the column and the sample was fractionated (2

minute intervals, 3.1 ml) in 13 x 100 mm tubes (Fisher-Scientific, Ottawa, ON, CA). The protein content of each fraction was read at an optical density (O.D.) of 280 nm (Kim, Dunn, & Dunn, 1988). The second peak (660 kD) was collected and dialyzed in 1 L of Fisher W5-4 water for 4-6 hours, (HPLC grade; Fisher-Scientific, Ottawa, ON, CA) three times, with one dialysis period being overnight. Tg was concentrated using an Amicon ultrafiltration cell (Amicon, Danvers, MA, USA) to ~1 mg/ml, placed in Eppendorf tubes (1 ml/tube) and put into a SpeedVac concentrator SVC100H (Savant Instruments Inc., Farmingdale NY, USA) to lyophilize the Tg. Once lyophilized, the Tg was placed in –20 °C.

2.2.2 Preparation of highly iodinated Tg (HI-Tg)

Highly iodinated Tg (HI-Tg) was prepared in our lab using Iodo-beads, following the manufacturer's instructions (Pierce Instruction Manual, Rockford, IL, USA). First, each bead was washed with 500 µl of PBS and dried on filter paper. In a (16 x 100 mm) glass culture tube containing 200 µl of PBS one bead was added. To the same tube, 20 µl of NaI (2.5 mM stock) was added and incubated for 5 minutes. Then, 200 µg/200 µl of Tg was added to each tube and incubated for 30 seconds at room temperature while mildly agitating the tube. This procedure iodinates Tg so that it contains ~30-40 I^- atoms/monomeric subunit. After the incubation period, the solution was removed from the tube to stop the reaction and the bead was washed to ensure all the protein was

collected. The Tg solution was dialyzed 3 times with Fisher water (1L). The Tg was pooled and the I_2 content was determined, as described in section **2.14**.

2.2.3 Tg peptides

The sequences and amino acid coordinates of all peptides are shown in **Table 2.1**. Peptides p110, p1579, and p1826 were synthesized by Sigma-Aldrich (St. Louis, MO, USA). Peptides p2495 and p2694 were synthesized by Alberta Peptide Institute (Edmonton, AB, CA) and all other peptides (p117, Ip117, p179, Ip179, p226, p304, p610, Ip610, p681, Ip681, p757, Ip757, Ip1390, p1931, Ip1931, p 2026, Ip2026, Ip2529, p2540, Ip2540, p2617, & Ip2617) were synthesized by Dalton (Toronto, ON, CA). Each peptide was purified (to > 80%) by HPLC and analyzed by mass spectroscopy (**Table 2.1**).

Table 2.1 Tg peptides

Number	a.a. coordinates	Peptide synthesized	Peptide denotation	
1	107-121	VQCDLQRVQ	p110	
2	121-130	VQCW**CVDTE**GMEVYGT	p117	
3	121-130	VQCW**CVDTE**GMEVY(I)GT	Ip117	
4	182-192	NTTDMMIFDLIHNYNR	p179	
5	182-192	NTTDMMIFDLIHNY(I)NR	Ip179	
6	228-239	LAETGL**ELLLD**EIYDTI	p226	
7	306-316	GHYQTV**QCQTE**GMCW	p304	
8	612-620	QCYAGE**CWCVD**SRGK	p610	
9	612-620	QCY(I)AGE**CWCVD**SRGK	Ip610	
10	684-689	SECY**CVDTE**GQVIP	p681	
11	684-689	SECY(I)**CVDTE**GQVIP	Ip681	
12	758-765	PHEQVFEWYERW	p757	
13	758-765	PHEQVFEWY(I)ERW	Ip757	
14	1393-1404	LHLDSKTFSADTTLY(I)FL	Ip1390	
15	1577-1591	LVQCLTDCANDEA	p1579	
16	1823-1837	GDMATELFSP	p1826	
17	1935-1942	KVVLNDKVNNFYTRL	p1931	
18	1935-1942	KVVLNDKVNNFY(I)TRL	Ip1931	
19	2029-2035	GSE**DTEVH**TYP	p2026	
20	2029-2035	GSE**DTEVH**TY(I)P	Ip2026	
21	2495-2511	GLINRAKAVKQFEESQG	p2495	mTgP1
22	2532-2543	EDSDARILAAAVWY(I)Y(I)SL	Ip2529	
23	2542-2552	VWYYSL**EHSTD**DYAS	p2540	
24	2542-2552	VWY(I)Y(I)SL**EHSTD**DY(I)AS	Ip2540	
25	2619-2626	SAYQG**QFSTE**EQSL	p2617	
26	2619-2626	SAY(I)QG**QFSTE**EQSL	Ip2617	
27	2694-2710	C(Acm)SFWSKYIQTLKDADGAK	p2694	TgP2 **

2.3 Culture media and cell lines

Lymphocytes were cultured in complete medium which consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Invitrogen, Grand Island, N.Y., USA), 10% fetal bovine serum [(FBS)(Cansera, Rexdale, ON, CA)], 5 x 10^{-5} M 2-mercaptoethanol (2-ME), and 20 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA). Bone marrow-derived dendritic cells (BM-DC) were cultured in complete medium which consisted of RPMI-1640 medium (Gibco Invitrogen, Grand Island, N.Y., USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS, 5 x 10^{-5} M 2-ME, and 10% supernatant from a cell line (X63Ag8; received as a gift from B. Stockinger, National Institute of Medical Research, London, U.K.) transfected with the murine granulocyte, macrophage, colony stimulating factor (GM-CSF) gene (Karasuyama & Melchers, 1988; Zal *et al.*, 1994). The IL-2-dependent CTLL line (Gillis & Smith, 1977) was purchased from American Type Culture Collection, Manassas, VA, USA. The cells were cultured in complete DMEM +10% IL-2-containing supernatant supplemented with methyl-α-D-mannopyranoside (50 mM final; Sigma, St. Louis, MO, USA). This supernatant was produced by culturing naïve (6 week old) rat splenocytes in complete media and concanavalin A (conA; 5 μg/ml) for 48 hours in a flask at 37 °C. At the end of the incubation period, the supernatant was decanted and supplemented with methyl-α-D-

mannopyranoside (50 mM) to inactivate any free conA. The supernatant was then filter-sterilized and stored at -20 °C.

2.4 Generation of $CD4^+$ T cell hybridomas

Three $CD4^+$ T cell hybridomas, 4A6.3, 10C1.1, and 1H7, were generated in vitro in our laboratory by immunizing CBA mice with 100 nmol of Tg peptide (I-p117, I-p304, and I-p1931, respectively). Seven days later, the mice were sacrificed, caudal, lumbar and inguinal lymph nodes were removed and single cell suspensions were made by passing the lymph node tissue through a sterile stainless steel wire mesh (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured (4×10^6 cells/ml) in complete DMEM in 1 ml aliquots in Multidish 24 well plates (Nunc, Roskilde, Denmark) in the presence of 10 μg/ml of Tg peptide. After 4 days of culture, the peptide primed lymphocytes were harvested, washed twice and placed in serum-free DMEM. The lymphocytes were fused with BW5147$\alpha^-\beta^-$ cells (White et al., 1989), a gift from P.Marrack (Howard Hughes Medical Institute, Denver, CO, USA), using 50% polyethylene glycol (PEG; Sigma-Aldrich, St. Louis, MO, USA) at a 1:2 ratio of T cells to tumor cells.

In a sterile 50 ml polypropylene centrifuge tube (Corning Inc., Corning, NY, USA), cells were mixed at the required ratio to get a concentration of 2.5×10^5 total cells/ml and spun at 1200 rpm for 5 minutes. The cells were washed twice and the supernatant was discarded. The tube was held on an angle and 1 ml of prewarmed 50% PEG was added drop-by-drop to the pellet over 1 minute, agitating gently after each drop. Next, 2 ml of

prewarmed serum-free DMEM was added over two minutes, agitating gently after each drop and 8 ml of prewarmed serum free DMEM was added over a 5 minute period, agitating gently after each drop. The tube was inverted several times to resuspend the pellet, the cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was discarded. The pellet was resuspended once with 10 ml of prewarmed DMEM + 20% FBS and more DMEM + 20% FBS was added until the cells were set at a concentration of 2.5 x 10^5 cells/ml. The cells were placed in a 96 well flat bottom plate (100 µl/well; Corning Inc, Corning NY, USA) and placed at 37 °C in a 10% CO_2, 90% air humidified incubator. Twenty-four hours later, 100 µl of 2x hypoxanthine-aminopterin-thymidine (HAT; Sigma-Aldrich, St. Louis, MO, USA) –containing DMEM + 20% FBS was added to each well. The cells were monitored daily and fed approximately every 5 days with 100 µl of 1x HAT-containing complete media (hybrids appeared between 7-21 days). When the cells reached ½ - ¾ confluency, they were transferred (1 ml aliquots) to a sterile Falcon multiwell (6 well) plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in 1x HAT medium. After 4-5 days, the HAT media was replaced with 1x hypoxanthine-thymidine (HT; Sigma-Aldrich, St. Louis, MO, USA) media for at least 2 cycles and then to DMEM + 10% FBS. Cells in wells that reached 75% confluency were tested for reactivity against the specific Tg peptide. To test for reactivity, an activation assay was preformed as described in section **2.13**. The antigen-specific hybridomas were cloned by limiting dilution at 0.3 cell/well using 1% syngeneic red blood cells (RBC) as filler cells as described in section **2.5** (Rao *et al.*, 1994).

2.5 Subcloning of T cell hybridomas by limiting dilution

The cells were harvested and washed 2 times with DMEM. Cells were resuspended in DMEM + 20% FBS, counted and set at a starting concentration of 3 x 10^4 cells/ml. The cells were serially diluted ten-fold until the final concentration was 3 cells/ml (0.3 T cells/well). Blood from a CBA/J mouse was collected (0.3 ml) and the RBC were used as filler cells. The RBC were washed once in 10 ml of DMEM, resuspended in DMEM + 20% FBS, and added to the final tube of cells (1% syngeneic RBC as filler cells). In a 96 well flat bottom plate, 100 µl of the RBC/cell mixture was added to each well and incubated at 37 °C in a 10% CO_2, 90% air humidified incubator. Seven and 12 days later, 50 µl of DMEM + 20% FBS was added to each well. After 14 days, if growth was evident, the cells were transferred into a 24 well plate. Once the cells reached 75 % confluency in the well, they were tested for reactivity against 1 µM of their specific Tg peptide. This method is described in section **2.13**.

2.6 Induction of experimental autoimmune thyroiditis (EAT)

CBA/J mice were immunized s.c. in the back of the neck and base of the tail with 100 µl of emulsion containing 200 µg of Tg in CFA (with *Mycobacterium butyricum*; Difco Laboratories, Detroit, MI, USA) on day 0. Half of the mice were boosted on day 21 with 100 µg of Tg in IFA (Difco Laboratories, Detroit, MI, USA) (Chronopoulou & Carayanniotis, 1992). On days 7, 21, 28, 35, and 42, the mice were sacrificed; thyroids

were removed, sectioned, stained, and analyzed for mononuclear cell infiltration as described in section **2.10**.

2.7 Induction of iodine-accelerated spontaneous autoimmune thyroiditis (IA-SAT)

NOD.H2^{h4} mice were given water containing 0.05% NaI (Sigma-Aldrich, St. Louis, MO, USA) for a period of 8 weeks, starting at 6-8 weeks of age. After 8 weeks, mice were euthanized and thyroids were removed, sectioned, stained and analyzed for mononuclear cell infiltration as described in section **2.10** (Rasooly *et al.*, 1996).

2.8 T cell proliferation assays

Seven days after immunization with Tg in CFA, draining lymph nodes (LN; inguinal, brachial, and axillary) were collected and single cell suspensions (LNC) (4 x 10^5 cells/well/100 µl) were made. LN cells (LNC) were cultured with Tg (100 µg/ml) or Tg peptides (10 µM), in 200 µl final volume in 96-well flat bottom plates. On days 21, 28, 35, and 42, T cell proliferation assays were performed using RBC lysed-splenocyte suspensions (8 x 10^5 cell/well) in 200 µl final volume. For T cell proliferation assays using NOD.H2^{h4} cells, all mice were sacrificed after 8 weeks on normal or NaI-water and thyroid draining cervical LN (TDCLN) or spleen were removed. Whole lymphocyte (4 x 10^5 cells/well/100 µl), splenocyte (8 x 10^5 cells/well/100 µl) and enriched splenic CD4$^+$ T cell populations were cultured in the presence or absence of Tg (100 µg/ml), I-Tg (32.5

μg/ml) or Tg peptides (10 μM). In cultures using enriched populations of $CD4^+$ T cells (10^5 cells/well/50 μl), syngeneic mitomycin-C (Sigma-Aldrich, St. Louis, MO, USA)-treated splenocytes (3 x 10^5 cells/well/50 μl) were used as an APC. All cells were cultured in complete DMEM and placed at 37 °C in a 10% CO_2, 90% air humidified incubator for 4 days. Eighteen hours before harvesting, 1 μCi of ^{3}[H]-thymidine (6.7 Ci/mol; PerkinElmer, Boston, MA, USA) was added to each well in 25 μl of medium (Lehmann *et al.*, 1992). The cells were harvested and thymidine incorporation was measured using a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter. Stimulation indices (SI) were measured as: (cpm in the presence of antigen)/(cpm in the absence of antigen) (Chronopoulou *et al.*, 1992).

2.9 Separation of $CD4^+$ T cells from spleen

Single cell suspensions were made from the spleens of NOD.$H2^{h4}$ mice and enriched $CD4^+$ T cell populations were negatively selected using an EasySep mouse $CD4^+$ T cell enrichment kit, following the manufacturer's instructions (Stem Cell Technologies Inc., Vancouver, BC, CA). This method specifically labels unwanted cells (non-$CD4^+$ T cells) with dextran-coated magnetic nanoparticles by using biotinylated antibodies against cell surface antigens expressed on the unwanted cells. The bispecific Tetrameric Antibody Complex (TAC) recognizes both biotin and dextran and therefore, anti-biotin Ab binds to biotin on the unwanted cells and anti-dextran Ab binds to dextran on the magnetic iron

nanoparticles. These labeled cells become trapped on the sides of the tube when place into the magnetic field and allow target cells to be separated.

Briefly, RBC-depleted splenocytes were washed with PBS and placed in PBS + 5% normal rat serum at a concentration of 10^8 cells/ml in a 12 x 75 mm sterile Falcon tube (Fisher-Scientific, Ottawa, ON, CA). Rat serum was used to prevent non-specific binding of rat Ab to mouse cells. Next, 50 µl of mouse-$CD4^+$ enrichment cocktail was added and the cells were incubated in the dark at 4 °C for 15 minutes. Next, 100 µl of biotin selection cocktail was added and the cells were incubated for 15 minutes in the dark at 4 °C. After 15 minutes, 50 µl of magnetic nanoparticles were added to the cells and they were incubated in the dark for 15 minutes at 4 °C. The mixture was topped up to 2.5 ml with PBS + 2% FBS and placed in an EasySep magnet for 5 minutes to attract the magnetically labeled cells to the sides of the tube. The magnet containing the tube was inverted for 2-3 seconds and the $CD4^+$-enriched cells were poured into a new sterile tube and used in T cell proliferation assays.

2.10 Histology

EAT was induced in CBA/J mice by immunization with Tg in CFA, as described in section **2.6**. The thyroid glands were removed, still attached to the trachea, and placed in 10% formalin (Fisher-Scientific, Ottawa, ON, CA). The lobes of the thyroid were dissected from the trachea, embedded in methacrylate, and sectioned at 3.0 µm intervals. The sections were placed on slides, 7 sections per slide, stained with haematoxylin and

eosin (H&E), and analyzed for mononuclear cell infiltration. Infiltration indices (I.I) were scored as follows: 0 = no infiltration, 1 = interstitial accumulation of cells between two or three follicles; 2 = one or two foci of cells, the size of a follicle; 3 = extensive infiltration, 10-40% of total area; 4 = extensive infiltration 40–80% of total area; and 5 = extensive infiltration >80% of total area (Verginis *et al.*, 2002).

2.11 Enzyme-linked immunosorbent assays (ELISA)

Upon sacrifice, blood was collected by heart puncture and spun at 1200 rpm for 10 minutes. The serum was collected, stored at -20 °C, and used to detect the presence of specific IgG Ab by ELISA. Disposable polyvinyl chloride plates (Dynex Technologies Inc., Chantilly, VA, USA) were coated overnight (100 µl/well) at 4 °C with 10 µg/ml of Tg, HI-Tg , or 1 µM of Tg peptide diluted in carbonate buffer, pH 9.6 (Sigma-Aldrich, St. Louis, MO, USA). The plates were washed with PBS and blocked with 0.1% BSA + PBS (200 µl/well) for 1 hour at room temperature. Then the plates were washed with PBS-Tween (PBS-T; Sigma, St. Louis, MO, USA) and 0.1% BSA + PBS-T was added (100 µl/well) to all wells except the first row. Sera samples at an optimum starting dilution were added (100 µl/well) to the first row, serially diluted 2-fold, and incubated for 1 hour at room temperature. The plates were washed three times with PBS-T, and a secondary antibody, goat anti-FC of mouse IgG (100 µl/well; Sigma-Aldrich, St. Louis, MO, USA) diluted at 1/2000 was added. The plate was incubated in the dark for one hour. The plates were washed three times with PBS-T and 100 µl of substrate solution (1

mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer; Sigma, St. Louis, MO, USA) was added to each well and incubated for 30 minutes. The absorbance of the p-nitrophenylate product was measured at 405 nm using a Vmax, kinetic microplate reader (Molecular Devices, Sunnydale, CA, USA) (Chronopoulou *et al.*, 1992).

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

Bone marrow (BM) was extracted from the tibia and fibia of NOD.H2^{h4} mice, and RBC were lysed with Tris-NH_4Cl (0.161M Tris-NH_4Cl; 0.1ml packed cells/ml Tris-NH_4Cl) for 2 minutes, under-layed with FBS, and centrifuged for 10 minutes at 1200 rpm. The resulting cells were washed 3 times with RPMI-1640 medium and were seeded at 2 x 10^6 cells/100 mm Petri dish (Corning Inc, Corning NY, USA). The cells were cultured in 10 ml R10 medium (RPMI-1640 + 10% FBS) + 10% GM-CSF and placed at 37 ºC in a 5 % CO_2, 95% air humidified incubator. On day 3, 10 ml of R10 + 10% GM-CSF was added to each plate. On days 6 and 8, half of the culture supernatant was removed, centrifuged, the pellet was resuspended in fresh R10 + 10% GM-CSF media, and the cells were placed back into their original dishes. After 10 days of culture, the nonadherent cells were gently dislodged by pipetting and used in activation assays (Lutz *et al.*, 1999).

2.13 Hybridoma T-cell activation assay

Cloned hybridoma T cells (10^5 cells/well, 50 µl/well) and BM-DC (10^5 cells/well, 50µl/well) were harvested, washed 3 times, and cultured for 24 hours in the presence or absence of 100 µl of 1 µM Tg, I-Tg, or Tg peptides (specific or control peptide). After 24 hours, 100 µl of supernatant was removed from each well, put into a new 96 well flat bottom plate, and placed at -20 °C for overnight. Frozen supernatants were later thawed and their IL-2 content was measured by the proliferation of CTLL cells. The CTLL cells were washed 4 times (to remove any leftover IL-2) and adjusted to 10^5 cells/ml. The cells (10^4 cells/well) were placed in wells containing either supernatant, complete DMEM (negative control), or complete DMEM + 10% Con A supernatant (positive control) and incubated for 24 hours at 37 °C, in a 10% CO_2, 90% air humidified incubator (Dai *et al.*, 1999). Six hours before harvesting, 1µCi of ^{3}[H]-thymidine was added to each well, the cells were harvested, and ^{3}[H]-thymidine incorporation was measured as described in section **2.8**.

2.14 Determination of iodine content in Tg

Free iodine was removed by extensive dialysis and the Tg-incorporated iodine was measured using a modified nonincinerative technique based on the catalytic activity of iodine in the ceric (Ce)-arsenite (As) reaction (Palumbo *et al.*, 1982; Saboori *et al.*, 1993). Mouse Tg (16 µg) was dissolved in 50% glacial acetic acid (Baxter Corporations, Toronto, ON, CA) in a 3 ml cuvette (Fisher-Scientific, Ottawa, ON, CA) to give a final

volume of 0.4 ml. To the cuvette, 160 µl of H_2SO_4 (36.7 N; BDH Inc., Toronto, ON, CA) was added, the cuvette was sealed, mixed by shaking, and incubated at room temperature for 10 minutes. Next, 80 µl of 1 mM Br_2 (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated at room temperature for 15 minutes. After 15 minutes the cuvette was placed into the spectrophotometer and 80 µl of Ce/NH_4SO_4 solution (Acros Organics, Morris Plains, New Jersey, USA) was added immediately, followed by 0.8 ml of 10 mM $NaAsO_2$ (Fisher-Scientific, Ottawa, ON, CA). The reagents were immediately mixed and after 13 seconds the absorbance was read at an O.D. value of 410 nm. One minute later, another reading of the cuvette was taken. Spectrophotometric analysis of the reduction of Ce (IV) to Ce (III) by As (III) showed a discoloration of the yellow Ce ion to a colorless ion. The results were then compared to a standard curve of T_4 (Sigma-Aldrich, St. Louis, MO, USA) which was constructed using known concentrations of T_4 dissolved in a 99 vol of absolute methanol (Fisher-Scientific, Ottawa, ON, CA) and 1 vol of 30 % ammonium hydroxide (Sigma-Aldrich, St. Louis, MO, USA). The same procedure was used to determine the standard curve (Dai *et al.*, 2002). The standard curve was plotted as the mean O.D. (initial reading OD – final reading OD) per minute versus µM/400 µl of iodine per sample. For normal Tg the expected number of iodine atoms/monomer was between 13-26 and maximally iodinated Tg was expected to contain ~60-70 Iodine atoms/monomer.

CHAPTER 3

T CELL EPITOPE SPREADING IN MURINE EAT

3.1 ABSTRACT

EAT, the murine model of Hashimoto's thyroiditis, can be induced in CBA/J mice after challenge with Tg in CFA. To date, at least 23 non dominant pathogenic T-cell epitopes have been discovered within the large (2749 aa) Tg molecule (Carayanniotis, 2007). However, the concept of T-cell epitope spreading has not been tested in EAT, as it has been in other animal models of autoimmune disease. To test this, we immunized CBA/J mice with 200 μg Tg in CFA and 7, 21, 28, 35, and 42 days later, mice (2 mice per time point) were sacrificed and spleen, lymph nodes, and sera samples were obtained. Mononuclear cell infiltrates of the thyroid were detected at all time points tested, but, Tg-specific proliferative T-cell responses were detected only on day 7 after challenge. Screening against a panel of 18 non-dominant pathogenic Tg peptides and their iodinated analogues showed that a significant T-cell response to these Tg peptides could not be generated up to 42 days after challenge. These results suggest that T-cell epitope spreading does not occur in CBA/J mice 42 days after challenge with Tg in CFA.

3.2 INTRODUCTION

The phenomenon of T cell epitope spreading has been observed in many T cell-mediated diseases. One disease in particular, EAE, has been extensively studied in regard to T cell epitope spreading. Studies using this model have revealed that the T cell immune response in EAE is dynamic, shifting from the recognition of a single dominant peptide to other cryptic peptides as the disease progresses (Lehmann *et al.*, 1992). The diversification of the T cell repertoire due to T cell epitope spreading in EAE has suggested: 1) a mechanism for the pathogenesis of T-cell driven autoimmune disease and 2) a possible therapeutic approach to prevent T-cell driven autoimmune disease before T cell epitope spreading occurs (Lehmann *et al.*, 1992). Lehmann *et al.* (1992) suggest that by inactivating the first wave of peripherally primed effector T cells this will prevent second-wave priming to autoantigens and prevent T cell epitope spreading (Lehmann *et al.*, 1992). Tg-induced EAT, another T cell-mediated disease, is characterized by the destruction of the thyroid gland by mononuclear cell infiltration. Like EAE, Tg-induced EAT does not occur spontaneously, requiring an immunization with Tg in CFA (Rose & Witebsky, 1956). Tg, the largest autoantigen in EAT, is composed of 2749 a.a. (Dunn & Dunn, 2000). Since the discovery of Tg by Roitt *et al.* (Roitt *et al.*, 1956), a total of 23 known pathogenic Tg peptides have been mapped within the huge molecule (Carayanniotis, 2007). Unfortunately, a dominant Tg peptide has yet to be discovered in the Tg molecule. Of the 23 Tg peptides mapped, all are immunopathogenic, but cryptic (Carayanniotis, 2007). Therefore, Tg-primed T cells are not activated by Tg peptides and

Tg peptide-primed T cells are not consistently activated by Tg *in vitro*. To date, there are three different categories of Tg peptides: 1) noniodinated peptides, 2) iodinated peptides containing hormonogenic sites, and 3) iodinated peptides containing iodotyrosyls (Li & Carayanniotis, 2006; Carayanniotis 2007). It is known that smaller doses of Tg peptide are required (50-200 nmol) to exert similar pathogenic effects on T cells than whole Tg (20-100 μg/61-303 μmol) (Carayanniotis, 2007). In order to make our results valid and reliable we used equimolar concentrations of whole Tg and Tg peptide to ensure that there were an equal number of molecules of each substance used when testing T cell reactivity.

In our study, we wanted to determine if T cell epitope spreading occurs in Tg-induced EAT in CBA/J mice. We hypothesized that over the course of 6 weeks, CBA/J mice challenged with Tg in CFA will show spreading of the immune response to cryptic Tg peptides. We also wanted to determine if boosting had an effect on T cell epitope spreading in Tg-induced EAT in CBA/J mice because boosting is an essential requirement for the optimal induction of EAT.

3.3 RESULTS

3.3.1 Induction of EAT in CBA/J mice

CBA/J mice were immunized subcutaneously with 200 μg Tg in CFA and 2 mice per time point were sacrificed on days 7, 21, 28, 35, and 42. Thyroids were removed and placed in formalin, histologically sectioned and stained, and scored for mononuclear cell infiltration. In the first experiment (days 7-42), all 10 mice tested had mononuclear cell infiltration in the thyroid gland ranging from 1-3 **(Table 3.1)**. Also, there was no significant difference between early and late time periods with respect to mononuclear cell infiltration in the thyroid gland ($p > 0.05$). In the second experiment **(Table 3.2)**, 13/14 mice developed thyroiditis with an I.I. of 1-4. These data confirmed that EAT was successfully induced in all mice through immunization with Tg in CFA and that the time of sacrifice did not have an effect on the development of disease **(Figure 3.1)**.

Table 3.1. Induction of EAT in CBA/J mice after receiving a single injection of 200 µg of Tg in CFA.

	Direct induction of EAT				
	Infiltration index (I.I.)				
Day	0	1	2	3	No. of mice with EAT
7	0	1	0	1	2/2
21	0	1	0	1	2/2
28	0	2	0	0	2/2
35	0	1	0	0	1/1*
42	0	1	1	0	2/2

*N/D for one mouse

Thyroids were histologically sectioned, stained with H&E and infiltration indices (I.I.) were scored according to the level of destruction in the thyroid gland by mononuclear cell infiltration.

Table 3.2. Confirmation of EAT induction in CBA/J mice after receiving a single injection of 200 µg of Tg in CFA.

	Direct induction of EAT					
	Infiltration index (I.I.)					
Day	0	1	2	3	4	No. of mice with EAT
7	1	1	0	0	0	1/2
21	0	0	1	0	1	2/2
28	0	0	2	0	0	2/2
35	0	0	2	0	0	2/2
42	0	1	1	0	0	2/2
56	0	0	1	1	0	2/2
70	0	2	0	0	0	2/2

Thyroids were histologically sectioned, stained with H&E and infiltration indices (I.I.) were scored according to the level of destruction in the thyroid gland by mononuclear cell infiltration.

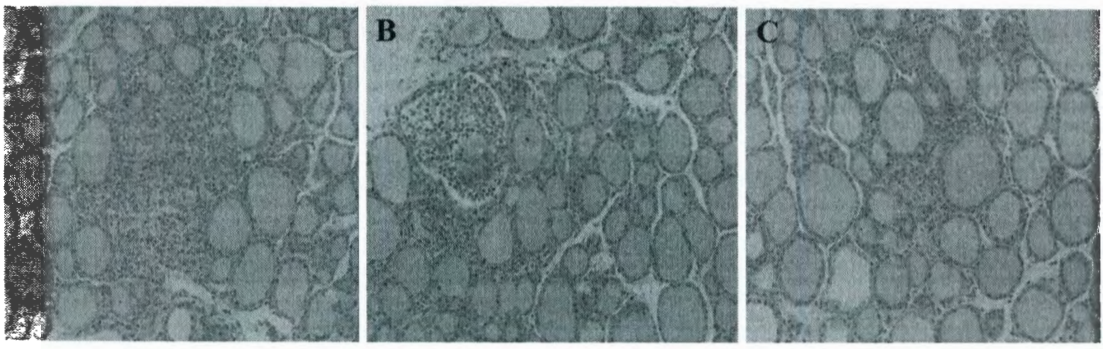


Figure 3.1. Histological appearance of mononuclear cell infiltration in thyroid glands of CBA/J mice after a single injection with Tg in CFA. Infiltration indices (I.I.) were scored as outlined in materials and methods. (A) I.I. = 3, day 7, 40X; (B) I.I. = 2, day 21, 40X; (C) I.I. = 1, day 42, 40X.

3.3.2 Antibody response to mTg

Serum was collected during each time period and ELISA was performed to measure the level of IgG Ab specific for mTg. Only individual sera, diluted at 1:200, from mice in the first experiment were tested. All mice given injections of Tg in CFA produced very high levels of IgG Ab in their serum against mTg compared to the sera taken from two naïve control CBA/J mice which did not produce Ab to mTg. The two control mice were sacrificed on day 42 of the experiment at 3-4 months of age and only serum was collected for testing. Also, the level of antibody increased as disease progressed. This was evident with an O.D. reading of 1 on day 7, to 3.5 on days 35 and 42 **(Figure 3.2)**. Sera were also tested against OVA in which no IgG Ab responses were detected, data not shown.

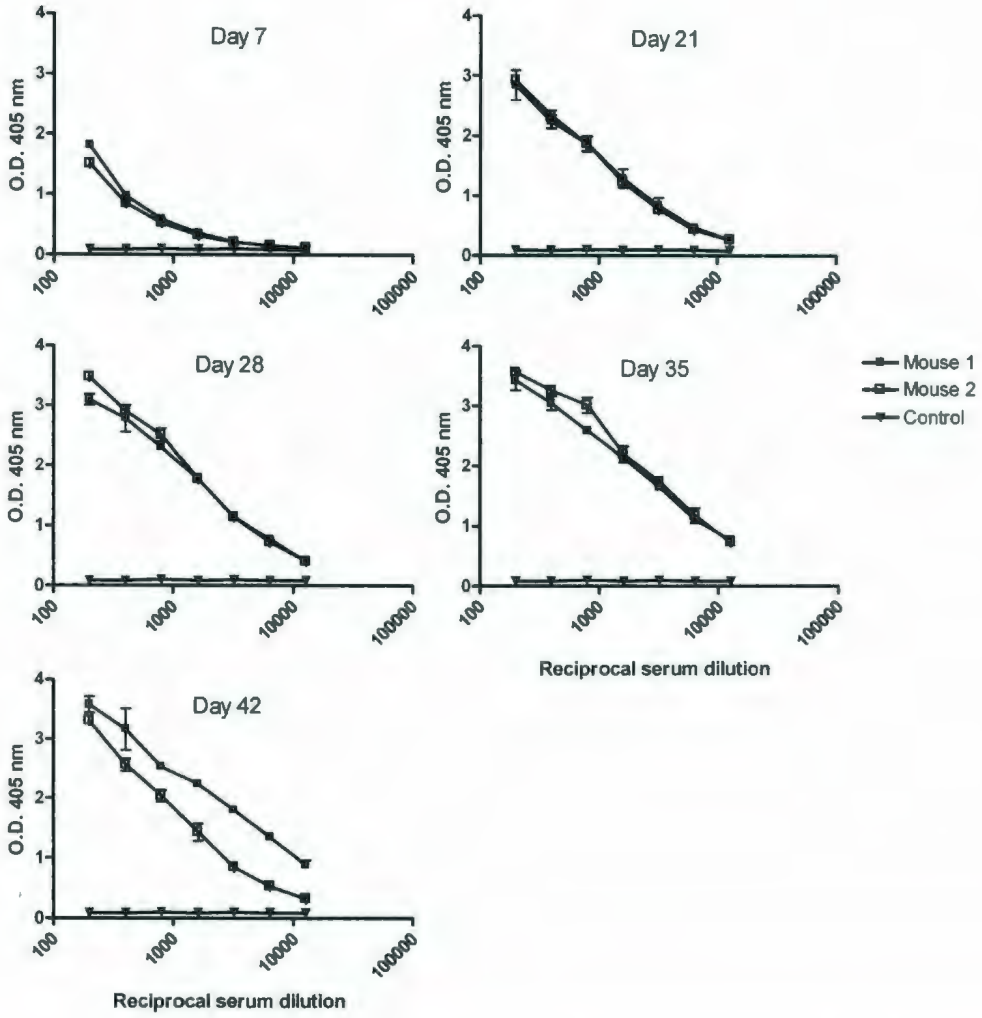


Figure 3.2. Serum IgG response against mouse Tg in CBA/J mice after a single challenge with Tg in CFA. Sera were collected from two naïve CBA/J mice (control) on day 42 and compared to ten CBA/J mice immunized with Tg in CFA on the days indicated (2 mice/group). Tg ELISA was performed to measure the specific IgG response to Tg. Individual sera were obtained from mice of Table 3.1 and were used at a starting dilution of 1:200. The O.D. was measured at 405 nm after 15 minutes.

3.3.3 T cell epitope spreading after a single s.c. challenge with Tg

CBA/J mice of Table 3.1 and 3.2 had their lymph nodes (day 7) or spleens removed at the time of sacrifice. T cell proliferation assays were conducted using whole populations of cells cultured in the presence or absence of 18 cryptic, but pathogenic Tg peptides and their iodinated analogues (Table 2.1) or whole Tg. T cell epitope spreading did not seem to occur in EAT, 6 weeks after immunization with Tg in CFA, because the T cells from CBA/J mice did not recognize any of the Tg peptides tested **(Figure 3.3)**. These results were confirmed, however, histology was not performed on the thyroids of these mice. Two later time points (days 56 and 70) were also tested to ensure that T cell epitope spreading was not delayed in this model **(Figure 3.4)**. In this second experiment the observed T cell responses to the Tg peptide p179 were not biologically meaningful because this Tg peptide also stimulated naïve CBA/J T cells **(Figure 3.5)** suggesting a non-specific effect. Collectively, these data provide no evidence for spreading of the immune response after challenge with intact Tg.

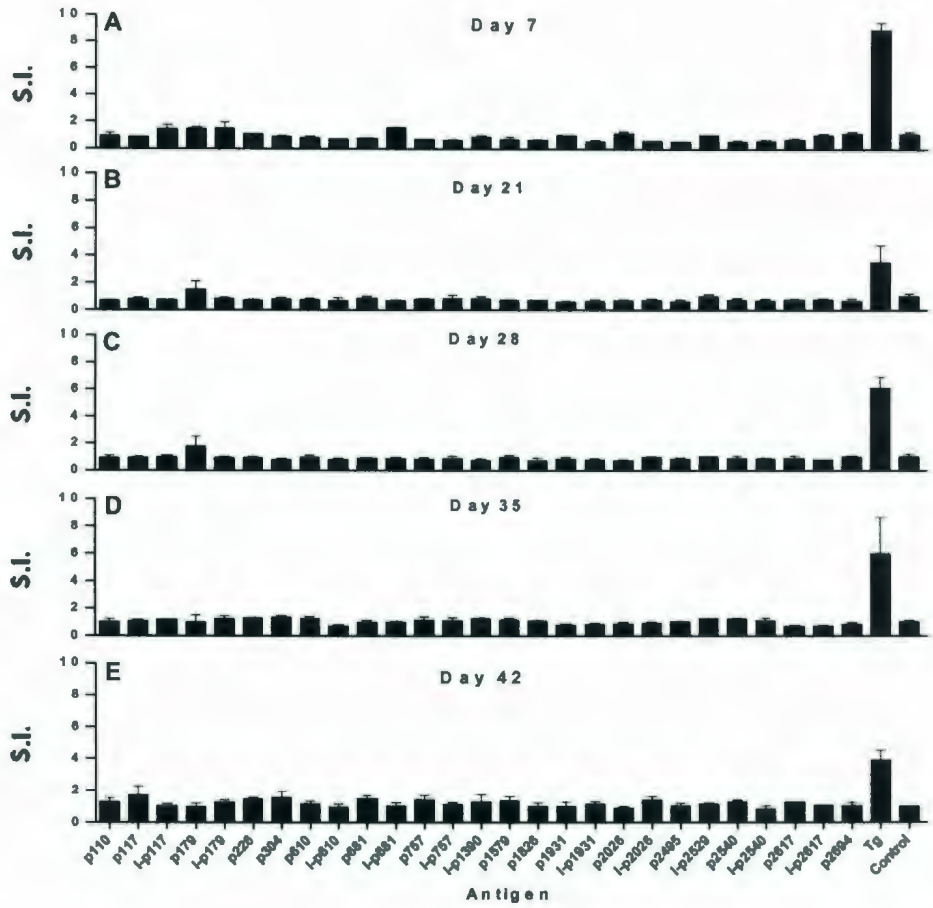


Figure 3.3. T cell epitope spreading does not occur in CBA/J mice after 6 weeks of priming with Tg in CFA. Female CBA/J mice (2 mice per time point) were immunized with Tg in CFA and sacrificed on the days shown. T cell proliferation assays were conducted using whole populations of spleen (8×10^5 cells/well; days 21, 28, 35, & 42) and lymph node cells (4×10^5 cells/well; day 7), cultured with a panel of 27 Tg peptides for 4 days. During the last 18 hours of culture, ^{3}[H]thymidine was added (1 μCi/well) and the cells were harvested after 96 hours of culture. S.I. were measured as follows: (cpm in the presence of Ag)/ (cpm in the absence of Ag).

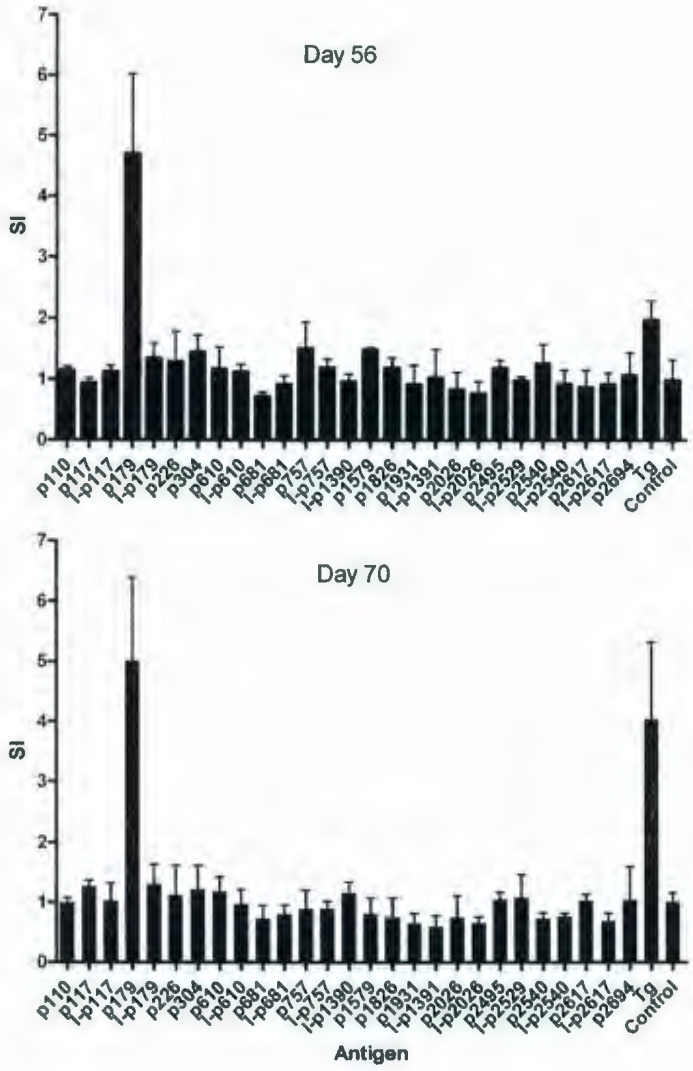


Figure 3.4. Lack of T cell epitope spreading 56 and 70 days post-immunization with Tg in CFA. T cell proliferation assays were conducted using splenocytes from female CBA/J mice immunized with a single injection of Tg in CFA and cultured in the presence or absence of a panel of Tg peptides and Tg.

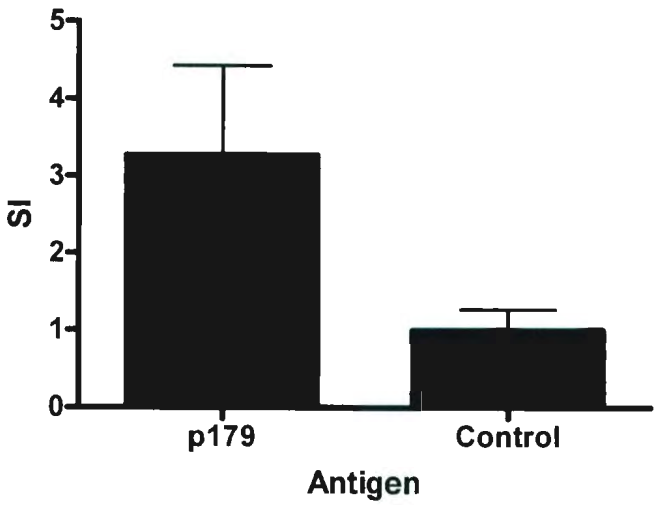


Figure 3.5. T cell reactivity to p179 in naïve CBA/J mice. Naïve splenocyte populations were cultured in the presence or absence of p179 for 4 days. S.I. were scored for p179 (S.I. = 3.3) and t-tests were performed ($p < 0.0018$).

3.3.4 T cell epitope spreading after two challenges with Tg

It is well known that EAT is routinely induced in CBA/J mice after two injections of Tg, one in CFA and the other in IFA (Charreire, 1989; Weetman & McGregor, 1984; Weetman & McGregor, 1994). We decided to test T cell epitope spreading in Tg-induced EAT in CBA/J mice after two challenges with Tg to determine if the number of challenges had an effect on T cell epitope spreading in this model. CBA/J mice were immunized with Tg in CFA on day 0 and boosted with Tg in IFA on day 21. On days 28, 35, and 42, two mice were sacrificed and thyroids, blood and serum were removed. T cell proliferation assays were conducted using whole splenocyte populations cultured in the presence or absence of 27 pathogenic, but cryptic Tg peptides and their iodinated analogs or whole Tg. According to our findings, we found that boosting had no effect on T cell epitope spreading in CBA/J mice. There was no significant T cell responses to any of the Tg peptides tested after 6 weeks of receiving their first immunization **(Figure. 3.6)**.

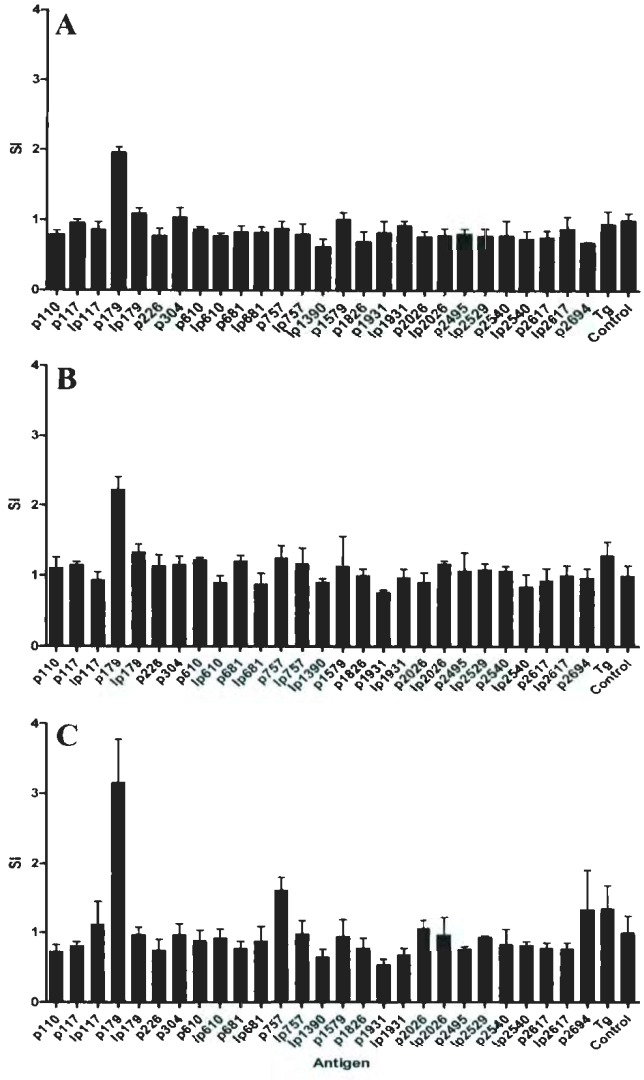


Figure 3.6. Lack of T cell epitope spreading in CBA/J mice after 2 challenges with Tg. CBA/J mice were immunized with Tg in CFA on day 0 and re-challenged with Tg in IFA on day 21. On days A) 28, B) 35, and C) 42, two mice were sacrificed and T cell proliferation assays were conducted using whole splenocyte populations cultured in the presence or absence of Tg and Tg peptides.

3.4 DISCUSSION

To date, T cell epitope spreading has not been tested in Tg-induced EAT, but it has been studied in other animal models of autoimmune disease. We tested T cell epitope spreading in Tg-induced EAT, using CBA/J mice immunized with Tg in CFA. One experiment revealed that T cell epitope spreading does not occur in EAT, up to 6 weeks after immunization with Tg in CFA in CBA/J mice. This was evident in the lack of T cell reactivity to a panel of 18 pathogenic, but cryptic Tg peptides and their iodinated analogues; this experiment was conducted three times with similar results. It is known that iodine can have variable effects on Tg peptides of known immunogenicity in regard to an increase, decrease or no change in antigenic or immunogenic response (Li & Carayanniotis, 2006). The I.I. scores and the production of IgG to mTg suggest that EAT was successfully induced in all mice tested, after immunization with Tg in CFA. Therefore, the lack of T cell epitope spreading in this model is not due to unsuccessful induction of disease. We also tested if boosting with Tg in IFA, three weeks after primary challenge might have an effect on T cell epitope spreading in Tg-induced EAT. These results were similar to the results obtained from the non-boosted mice, i.e. we found no evidence of T cell epitope spreading using the panel of 18 Tg peptides and their iodinated analogs. From these findings we conclude that boosting had no effect on T cell epitope spreading in CBA/J mice with EAT. Although T cell epitope spreading was not detected in Tg-induced EAT, 6 weeks post-injection, there was no T cell response to any of the 18 Tg peptides tested even after 56 and 70 days. The response to p179 was deemed

biologically insignificant because it occurred even with naïve CBA/J mouse T cells suggesting the presence of contaminants with stimulatory properties.

Although the results show that Tg-primed T cells do not recognize our panel of Tg peptides, we cannot rule out T cell epitope spreading in EAT as other unknown Tg peptides may be recognized. However, the lack of a T cell response to Tg after 21 days may indicate that if Tg is the autoantigen involved in initiating EAT, it may not be involved in maintaining this disease. The fact that CBA/J mice developed EAT shows that the lack of T cell epitope spreading was not due to lack of immunogenic stimulus, but that these peptides are not generated after the processing of Tg *in vivo*.

3.5 FUTURE DIRECTIONS

Tg is a large molecule and may contain many more T cell epitopes. Testing such epitopes as potential targets during the course of EAT may be required to determine if T cell epitope spreading occurs in Tg-induced EAT. Also, the discovery of a dominant Tg peptide would aid in testing T cell epitope spreading in this model. That way, one could monitor the spreading of the immune response from a dominant peptide to other cryptic Tg peptides during the progression of disease.

CHAPTER 4

THE ROLE OF Tg IN IODINE-ACCELERATED SPONTANEOUS AUTOIMMUNE THYROIDITIS

4.1 ABSTRACT

NOD.H2^{h4} mice develop iodine-accelerated spontaneous autoimmune thyroiditis (IA-SAT) after ingesting NaI in their drinking water for 8 weeks. IA-SAT causes the destruction of the thyroid by mononuclear cell infiltration and the production of thyroglobulin (Tg)-specific antibodies (Ab) in the serum. Tg, a target autoantigen in spontaneous autoimmune thyroiditis (SAT) and IA-SAT, can store available iodine in iodotyrosyl residues. In this study, we wanted to determine if our panel of Tg peptides are involved in the development and/or maintenance of IA-SAT in NOD.H2^{h4} mice. We tested whether T cells from NOD.H2^{h4} mice given 0.05% NaI in their drinking water for 8 weeks could recognize a panel of pathogenic, iodinated or non-iodinated Tg peptides when cultured *in vitro*. We found that while NOD.H2^{h4} mice develop IA-SAT and Tg-specific Abs, their lymph node, splenic and purified CD4^{+} T cells do not recognize our panel of Tg peptides revealing that these peptides are not involved in IA-SAT development. Although there is strong evidence that Tg is a target autoantigen for T cells in EAT and for B cells in IA-SAT, our results do not provide evidence for T cell reactivity against our panel of pathogenic Tg epitopes.

4.2 INTRODUCTION

NOD.H2^{h4} mice were derived by crossing NOD(H2^{g7}) mice with B10.A(4R)(H-2^{h4}) mice and then backcrossing the heterozygous progeny expressing the A^k gene with NOD mice for many generations (Podolin et al., 1993). After 6 generations, brother-sister matings occurred which resulted in the generation of NOD.H2^{h4} mice. These mice were shown to develop SAT after 6 months of age, but no diabetes (Podolin et al., 1993). However, 6-8 week old NOD.H2^{h4} mice placed for 8 weeks on drinking water with 0.05% NaI, develop SAT with accelerated onset and increased severity of IA-SAT as well as high Ab titers to mTg (Braley-Mullen *et al.*, 1999; Rasooly *et al.*, 1996).

Tg, the major autoantigen in autoimmune thyroiditis, is a large homodimeric molecule weighing ~660 kDa (Dunn & Dunn, 2000). An important feature of Tg is that it can incorporate and store available I_2 in the form of iodotyrosyl residues (Dunn, 1995) which are required in the formation of the thyroid hormones T_3 and T_4. Since the discovery of Tg, there have been a total of 23 pathogenic, but cryptic Tg peptides identified (Carayanniotis, 2007). Iodination of some of these Tg peptides has had various influences on their immunogenicity and antigenicity, whereby iodination either causes an increase, decrease or no change in the Tg peptides immunogenic response (Li & Carayanniotis, 2006). Quite a few of these peptides are A^k-restricted, but it remains unknown whether they are targets of the autoimmune response in NOD.H2^{h4} mice (they contain the A^k allele). Interestingly, highly iodinated Tg (HI-Tg) has been found to be more immunogenic than normal Tg, suggesting that it may harbor iodine-modified epitopes

(Dai *et al.*, 2002). Based on the above rationale we hypothesized that iodinated Tg peptides may be preferential targets in IA-SAT in NOD.H2^{h4} mice, and we proceeded to test their antigenicity at the B and T cell level.

4.3 RESULTS

4.3.1 Iodine-accelerated SAT (IA-SAT)

Male and female NOD.H2^{h4} mice (6 mice/group) were given 0.05% NaI water at 6-8 weeks of age for a period of 8 weeks and control mice (6 mice/group) received normal water. After 8 weeks, the mice were sacrificed and thyroids were removed. The thyroids were placed in 10% formalin and histologically sectioned and stained to determine the level of mononuclear cell infiltration in the gland. Histological analysis of the thyroids confirmed that NOD.H2^{h4} mice develop SAT at an accelerated rate after drinking NaI water for 8 weeks. All male NOD.H2^{h4} mice given NaI water developed thyroiditis with an I.I. of 4 **(Table 4.1 and Figure 4.1)**. Female mice on NaI water also developed thyroiditis, but it was less severe, ranging from an I.I. of 1-3, with one mouse not developing thyroiditis. In the male control group, thyroiditis was significantly lower with only 2 out of 6 males developing disease, the highest having an I.I. of 2. However, in the female control group, 4 out of 6 females developed thyroiditis and there was no significant difference in I.I. scores among the two female groups.

Table 4.1: NOD.H2^{h4} mice given 0.05% NaI water for 8 weeks develop IA-SAT.

	Infiltration Index (I.I.)[b]					
Mice[a]	**0**	**1**	**2**	**3**	**4**	**Total mice with SAT**
Male NaI	0	0	0	0	6	6/6
Female NaI	1	1	2	2	0	5/6
Male Controls	4	1	1	0	0	2/6
Female Controls	2	1	1	2	0	4/6

$^{**}p > 0.05$ (5/6 vs 2/6); $^{*}p < 0.05$ (6/6 vs 2/6 and 4/6)

[a]Male and female NOD.H2^{h4} mice at 6-8 weeks of age were given normal water or water containing 0.05% NaI for a period of 8 weeks. At 14-16 weeks of age, the mice were sacrificed and thyroids were removed, histologically sectioned, stained with H&E, and analyzed for mononuclear cell infiltration in the gland.

[b]Infiltration indices (I.I.) were scored according to the scale provided in Materials and Methods.

*t-test analysis of differences in severity of thyroiditis among males on NaI water and males on normal water, $p < 0.05$

**t-test analysis of differences in severity of thyroiditis among females on NaI water and females on normal water, $p > 0.05$

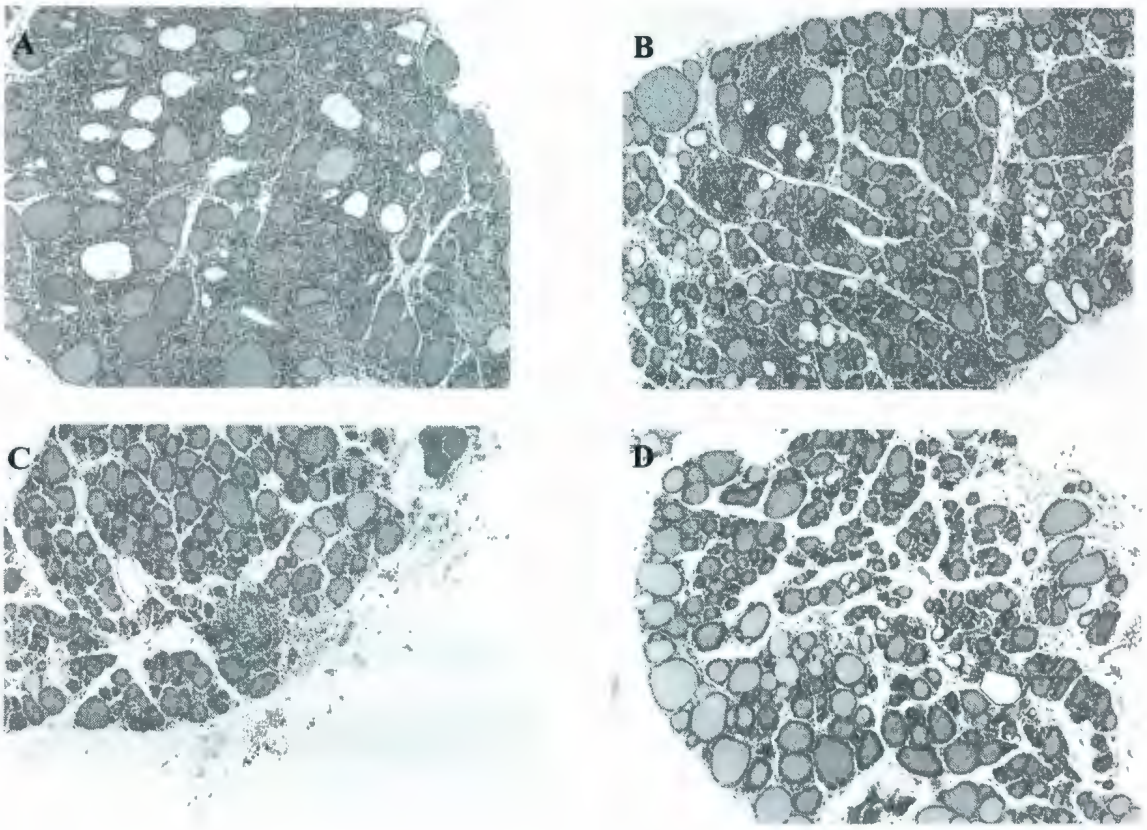


Figure 4.1. Iodine-accelerated SAT in the thyroids of male and female NOD.H2^{h4} mice given 0.05% NaI for 8 weeks. Mice were given 0.05% NaI water at 6-8 weeks of age. After 8 weeks on water, mice were sacrificed and thyroids were removed and placed in 10% formalin. The thyroids were histologically sectioned and stained with H&E. Infiltration indices were scored as described in Materials and Methods. A) I.I. = 4 (male), 100x; B) I.I. = 3 (female), 100x; C) I.I. = 1 (male), 100X; and D) I.I. = 0 (male), 100X.

4.3.2 Antibody response to mTg, HI-Tg, Tg peptides and iodinated analogs

Sera were collected from 14-16 week old NOD.H2^{h4} mice at time of sacrifice to assay for the presence of IgG to Tg and HI-Tg to determine if the I_2 content of Tg influences the antigenicity of Tg in IA-SAT. The pooled sera from each group were used at a starting dilution of 1:100. Our results show that male and female NOD.H2^{h4} mice on 0.05% NaI water for 8 weeks mounted equivalent IgG responses against Tg **(Figure 4.2 (A, B))** and HI-Tg **(Figure 4.2 (C, D))**. Control mice did not develop Tg or HI-Tg-specific Abs. Also, none of the mice produced Ab to OVA (data not shown) which was used as an antigen control.

We subsequently tested the sera from younger (6-8 week old) mice to determine if IgG Ab to Tg and HI-Tg were present. Sera from young 6-8 week old male NOD.H2^{h4} mice were collected and tested for IgG Ab to Tg and HI-Tg **(Figure 4.3)**. The results showed that there was very little Ab produced against Tg and HI-Tg compared to older 14-16 week old mice **(Figure 4.2 – 4.3)**. However, the level of IgG Ab to Tg and HI-Tg was the same **(Figure 4.3)**. These results suggested that there were no new determinants formed in Tg after the consumption of NaI water for 8 weeks in NOD.H2^{h4} mice because an increase in iodination of Tg did not affect its antigenicity. As a control, we decided to test the IgG response to a panel of Tg peptides and their iodinated analogs. NOD.H2^{h4} sera from male mice on NaI water for 8 weeks were tested for the production of IgG to 10 mouse Tg peptides and their iodinated analogs. The sera were pooled, 6 mice per group, and used at a final dilution of 1:20. Only IgG Ab levels 2 SD above the average

background were considered detectable responses. Our data showed that sera from NOD.H2^{h4} mice on normal and NaI water for 8 weeks contained IgG against 6 of the 20 Tg peptides (p117, I-p117, p304, I-p304, p2026,and I-p2617) **(Figure 4.4 (A))**. We chose to concentrate only on these 6 Tg peptides because IgG responses to the other Tg peptides tested were below or barely above the average background. Analysis of the IgG response against these 6 Tg peptides revealed that the ingestion of 0.05% NaI water did not significantly enhance the IgG response to our panel of Tg peptides and their iodinated analogs. In fact, increased iodine intake did cause a significant decrease in the level of IgG against the Tg peptides p2026 and I-p2617 and it had no effect on the level of IgG against the Tg peptides p117, I-p117, p304 and I-p304 **(Figure 4.4 (A))**. We also tested sera from young 6-8 week old NOD.H2^{h4} mice to determine if there is spontaneous Ab reactivity to the 10 Tg peptides and their iodinated analogs **(Figure 4.4 (B))**. We found that IgG responses were detected to 8 Tg peptides (p117, I-p117, p304, I-p304, I-p1931, p2026, I-p2540, and I-p2617), but at lower levels compared to older (14-16 week old) mice **(Figures 4.4 (A))**. Taken together, these results suggest that there is spontaneous Ab reactivity to some of the Tg peptides tested in NOD.H2^{h4} mice and that these levels are not enhance by the consumption of dietary iodine.

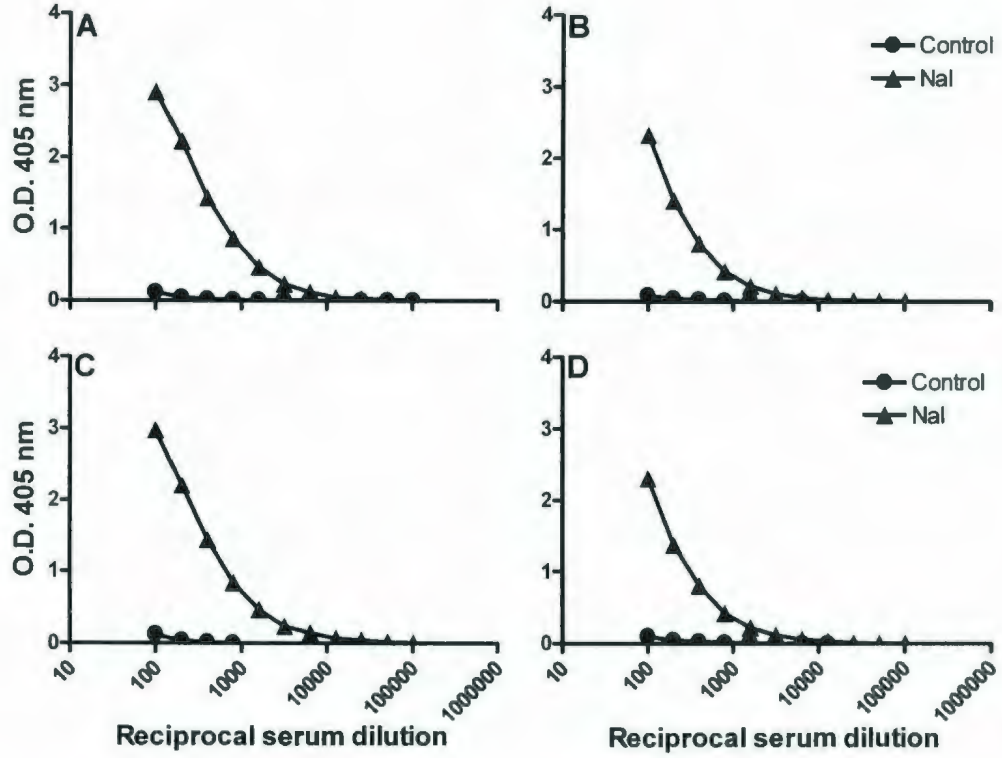


Figure 4.2. NOD.H2^{h4} IgG Ab response to Tg and HI-Tg. Sera were pooled from six male (A,C) and three female (B,D) NOD.H2^{h4} mice after 8 weeks on 0.05% NaI water or normal water. A starting serum dilution of 1:100 was used to measure the IgG response to Tg (A, B) and HI-Tg (C, D). The optical density (O.D.) was measured at 405 nm after 30 minutes. Responses to OVA were not detectable. Data are representative of two independent experiments. t-test analysis confirmed: male response to Tg, $p < 0.05$ (NaI vs Control); male response to HI-Tg, $p < 0.05$ (NaI vs Control); female response to Tg, $p < 0.05$ (NaI vs Control); and female response to HI-Tg, $p < 0.05$ (NaI vs Control).

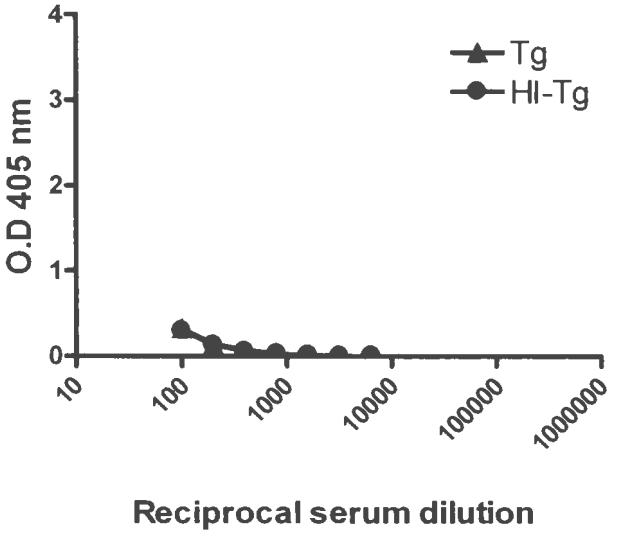


Figure 4.3. Serum IgG responses to Tg and HI-Tg from male, 6-8 week old NOD.H2^{h4} mice. Pooled sera from 6, 6-8 week old male NOD.H2^{h4} mice at a starting dilution of 1:100 were tested for the presence of IgG specific for Tg and HI-Tg. The O.D. was measured at 405 nm after 30 minutes.

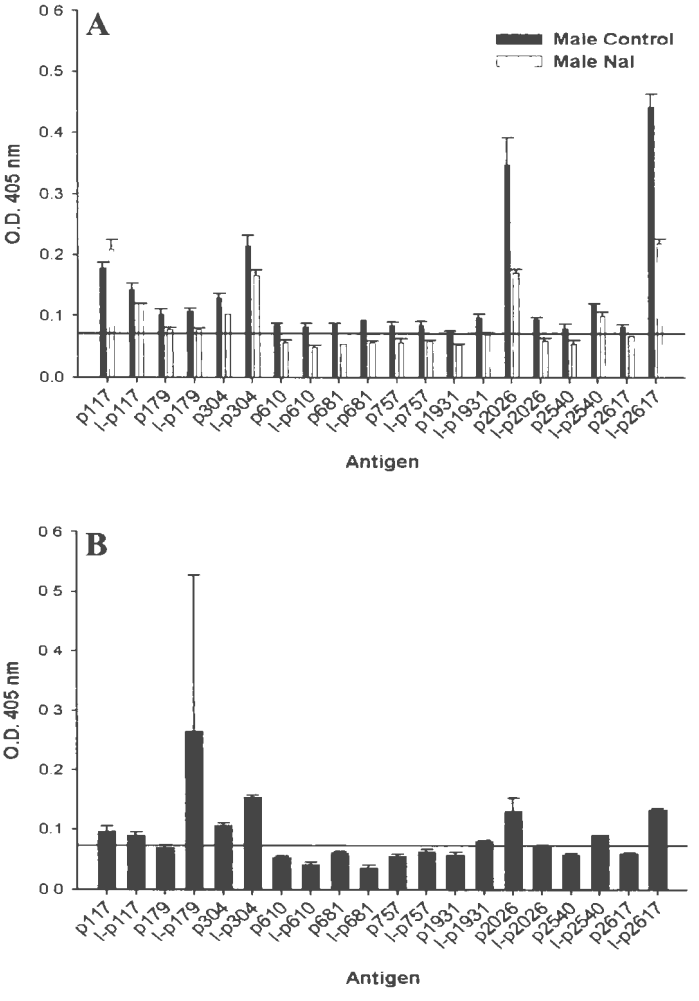
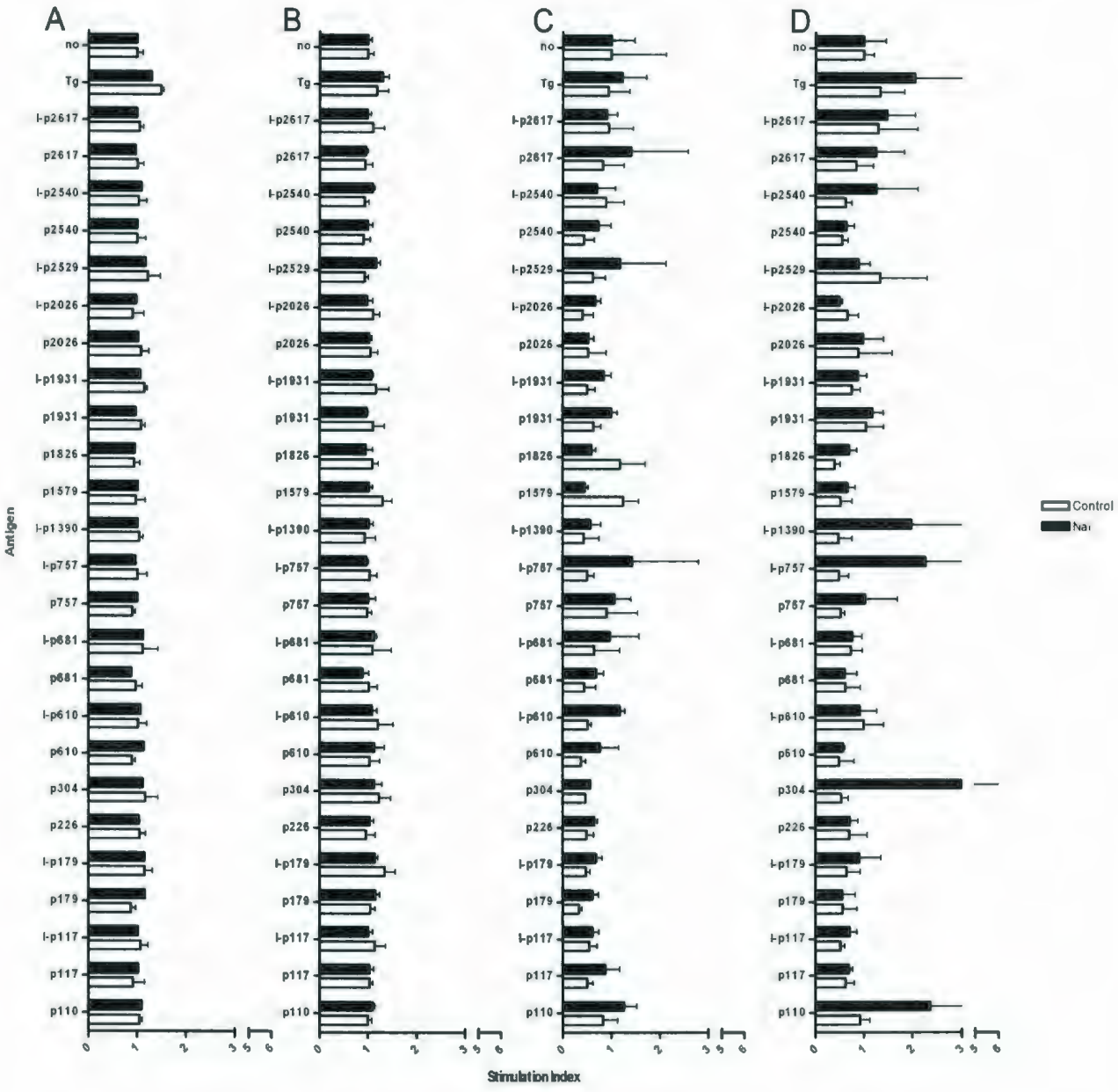


Figure 4.4. IgG response to Tg peptides and their iodinated analogs using male NOD.H2^{h4} mouse sera. Male, 12-14 week old (A) and naïve 6-8 week old (B) NOD.H2^{h4} mouse sera were collected from mice on normal and 0.05% NaI water for 8 weeks. The sera were pooled, 3 mice per group, and used at a 1:20 dilution. The O.D. was measured at 405 nm and read after 30 minutes. A response was deemed significant if it was 2 SD above the average background. The line in the figures represents the average background which ranges from 0.065 – 0.099 for the older mice and 0.066-0.077 for the younger mice.

4.3.3 T cell reactivity in IA-SAT in NOD.H2^{h4} mice

To date, it has not been reported whether NOD.H2^{h4} mice can mount Tg-specific T cell responses during SAT. Since Tg incorporates and stores available I_2 and HI-Tg has been shown to be more immunogenic in EAT than normal Tg (Dai *et al.*, 2002), we wanted to determine if T cells from NOD.H2^{h4} mice on NaI water for 8 weeks can recognize a panel of pathogenic Tg peptides and their iodinated analogs. We chose Tg peptides which were A^k-binders because NOD.H2^{h4} mice carry the A^k gene, making them susceptible to IA-SAT. Male and female NOD.H2^{h4} mice were given 0.05% NaI water or normal water for 8 weeks, starting at 6-8 weeks of age. After 8 weeks, mice were sacrificed and cells from spleens or thyroid-draining lymph nodes (TDLN) were collected and pooled (3 mice per group, male and female groups). T cell proliferation assays were conducted to measure the T cell reactivity against a panel of 18 A^k-binding, pathogenic Tg peptides and their iodinated analogs. A response was deemed significant if it was two SD above the background mean SI response. According to our findings, T cells from whole spleen or purified splenic CD4$^+$ T cells **(Figure 4.5)** did not significantly respond to any of the Tg peptides tested or to Tg. Also, T cells from TDLN populations did not respond to the Tg peptides, Tg or HI-Tg **(Figure 4.6)**. These data demonstrate that NOD.H2^{h4} mice cannot mount Tg-specific T-cell responses to our panel of Tg peptides and their iodinated analogs during IA-SAT.


A
B
C
D
no
Tg
I-p2617
p2617
I-p2540
p2540
I-p2529
I-p2026
p2026
I-p1931
p1931
p1826
p1579
I-p1390
I-p757
p757
I-p681
p681
I-p610
p610
p304
p226
I-p179
p179
I-p117
p117
p110
Antigen
Stimulation Index
Control

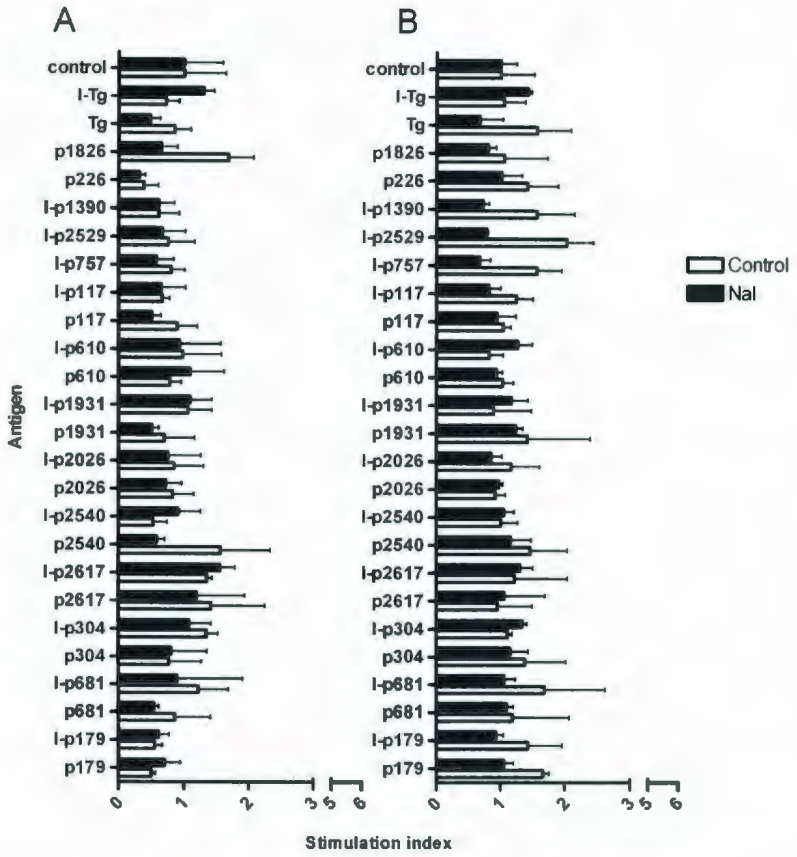


Figure 4.6. Responses of thyroid-draining lymph node cells to a panel of Tg peptides. (A) male and (B) female NOD.H2^{h4} mice were either given 0.05% NaI in their drinking water or normal water for 8 weeks, starting at 6-8 weeks of age. After 8 weeks, the mice were sacrificed and TDLN were removed, pooled (3 mice/group), and used in T cell proliferation assays. The cells were cultured in the presence or absence of Tg, HI-Tg, and a panel of Tg peptides for 4 days. During the last 18 hours of culture, ^{3}[H]thymidine was added. At the end of culture the cells were harvested and measured for thymidine uptake as previously described in Fig. 4.7. A response was deemed significant if it was 2 SD above the mean background SI value.

4.3.4. NOD.H2^{h4} BM-DC cannot process HI-Tg or Tg to generate I-p117, I-p304, & I-p1931

To determine if the lack of a T cell response to our panel of Tg peptides was due to a low frequency of T cells present in the spleen and TDLN of NOD.H2^{h4} mice on 0.05% NaI water for 8 weeks, we decided to test T cell clones for reactivity to specific Tg peptides. We hypothesized that HI-Tg (43.2 I atoms/monomeric subunit), but not normal Tg (15-20 I atoms/monomeric subunit), might be differentially processed and presented by NOD.H2^{h4} BM-DCs to generate the cryptic pathogenic Tg peptides I-p117, I-p304, and I-p1931 which will be recognized by A^{k}-restricted Tg peptide-specific T cell clones. BM-DC were generated from NOD.H2^{h4} mouse bone marrow and cultured in the presence of 10% GM-CSF for 10 days. On day 10, an activation assay was performed using BM-DC and three CD4^{+} T cell hybridoma clones specific for the Tg peptides I-p117, I-p304, and I-p1931. These cells were cultured in the presence or absence of Tg, HI-Tg, specific Tg peptide, and control Tg peptide. Hybridoma activation was assessed by measuring the IL-2 content in culture supernatants using a proliferation assay of the IL-2 dependent CTLL cells. It was found **(Figure 4.7)** that none of the hybridomas were activated upon culture with Tg, HI-Tg or control peptide (p304). Only their specific ligand as a free peptide (I-p117, I-p304, or I-p1931) could activate the hybridomas to secrete IL-2. These findings do not support the hypothesis that enhanced iodination of Tg facilitates the generation of these iodinated peptides during Tg processing *in vitro*.

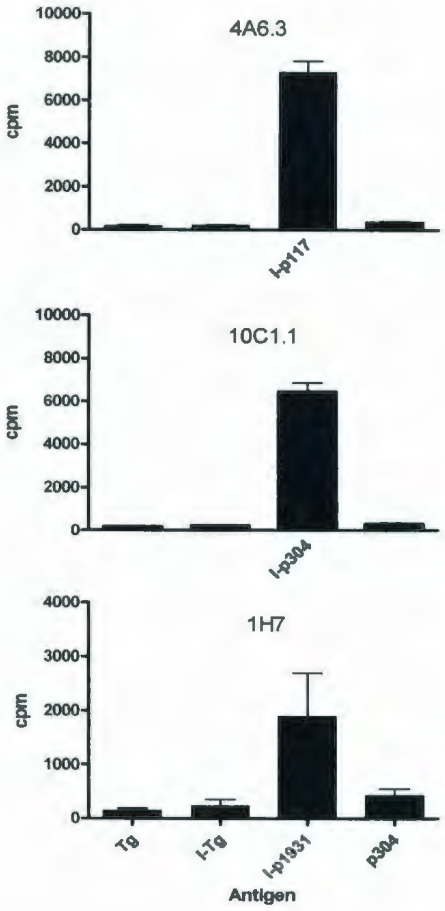


Figure. 4.7. CD4$^+$ T cell hybridomas specific for I-p117, I-p304, and I-p1931 are not activated by Tg or HI-Tg after processing by NOD.H2^{h4} BM-DC. The indicated T cell hybridoma clones (10^5 cells/well) were cultured with BM-DC (10^5 cells/well) in the presence or absence of Tg, HI-Tg, specific Tg peptide and control Tg peptide (1μM). A CTLL assay was performed as described in Materials and Methods to measure the IL-2 secretion by each hybridoma. Positive control wells containing CTLL + supernatant with IL-2 had a cpm of 19682, and the negative control wells containing CTLL alone was 376.

4.4 DISCUSSION

The ingestion of 0.05% NaI water for 8 weeks caused severe IA-SAT in both male and female NOD.H2^{h4} mice confirming the earlier results of Braley-Mullen *et al.* and Rasooly *et al.* (Braley-Mullen *et al.*, 1999; Rasooly *et al.*, 1996). However, we observed an increased incidence of SAT in female NOD.H2^{h4} mice given normal water for 8 weeks, leading to a similar incidence of SAT in female mice between the control and experimental groups. It is known that SAT incidence in NOD.H2^{h4} mice given normal water for 8 weeks is low (Rasooly *et al.*, 1996). The fact that 4/6 (67%) female mice given normal water for 8 weeks developed disease could be an indication that SAT may develop in our colony at a more accelerated rate than in other colonies. Braley-Mullen *et al.* documented the kinetics of SAT in NOD.H2^{h4} mice showing that mice on normal water for 8 weeks do not have mononuclear cell infiltration in their thyroid glands and that control mice rarely developed disease before the age of 6 months (Braley-Mullen, 1999). The kinetics of IA-SAT in our colony should be examined to determine if IA-SAT develops at a more rapid rate in our colony compared to other colonies. This may be the reason why we detect disease in female control NOD.H2^{h4} mice.

Sera were collected from all mice at the time of sacrifice to assay for the presence of IgG Ab to Tg and HI-Tg. We wanted to determine if an increase in dietary iodine influenced the antigenicity of Tg in IA-SAT. Our results suggested that both male and female NOD.H2^{h4} mice on NaI water for 8 weeks produced significant amounts of IgG Ab to Tg

compared to NOD.H2^{h4} mice on normal water for 8 weeks (Braley-Mullen *et al.*, 1999; Rasooly *et al.*, 1996). Also, both male and female NOD.H2^{h4} mice on NaI water for 8 weeks produced significantly higher amounts of IgG Ab to HI-Tg compared to NOD.H2^{h4} mice on normal water for 8 weeks. Further, we noticed that the level of IgG Ab against Tg was the same as the level of IgG Ab against HI-Tg for all groups of mice tested. These results were interesting because they did not provide evidence for the existence of iodine-modified epitopes being the target for autoantibodies in this model. Since it is known that excess iodine is incorporated and stored in the Tg molecule (Dunn, 1995), we assumed that the iodine consumed by NOD.H2^{h4} mice might be incorporated into Tg causing an increase in IA-SAT. If neodeterminants were generated at the B-cell level then we would expect to see a higher Ab response to HI-Tg than Tg. The detection of low levels of IgG Ab in the serum of 6-8 week old NOD.H2^{h4} mice shows that there are existing IgG Ab to Tg and HI-Tg in the serum, however these levels are significantly lower than mice on NaI water for 8 weeks ($p < 0.05$). These results suggest that an increase in dietary iodine is causing an increase in the level of IgG Ab against Tg and HI-Tg, but further experiments need to be conducted to determine what mechanisms are involved in this process and whether iodine is being incorporated directly into the Tg molecule.

We decided to look at the IgG response to 10 Tg peptides and their iodinated analogs to determine if an increase in dietary I_2 intake in NOD.H2^{h4} mice would prompt Ab production to these Tg peptides in the sera. We found that increased dietary intake of I_2

for 8 weeks did not enhance the IgG response to any of the Tg peptides tested when compared to the IgG response of control mice to these same Tg peptides. We did notice however, that the consumption of dietary I_2 either decreased the antigenicity or had no affect on the antigenicity of these Tg peptides. Analyzing the non-iodinated analogs revealed that control mice produced antibody to the Tg peptides p117, p304, and p2026, suggesting that there is "spontaneous" IgG production against these epitopes and that they are presumed to be on the molecular surface of Tg. In mice given NaI water for 8 weeks, we noticed relatively low and similar IgG responses to p304 and p117 respectively, and that the IgG level to p2026 was significantly lower compared to the controls. If I_2 modifies Tg, there is no effect on the anti-p117 and anti-p304 responses because I_2 atoms are not added at the vicinity of, or within these epitopes. Also, if I_2 modifies Tg, there is a diminished response to p2026 because I_2 atoms are added at the vicinity of, or within this epitope, therefore causing a blocking effect. If I_2 does not modify Tg, it is hard to explain the selective decrease of the IgG response to p2026 only. In the case of the iodinated analogs, we observed that control mice had "spontaneous" IgG production against the Tg epitopes I-p117, I-p304, & I-p2617. For the Tg peptides I-p117 and I-p304, we can conclude that I_2 atoms naturally occur at these sites and/or the addition of I_2 to these sites does not alter their recognition by B cells, as it appears by comparison to the response against the non-iodinated analogs (see above). For the Tg peptide I-p2617, we can conclude that this site may be "naturally" iodinated and dominant in comparison to the other sites tested. This is further suggested by the lack of response to p2617 in control

mice. In NOD.H2^{h4} mice that were given NaI water for 8 weeks, we noticed that the IgG response to I-p117 and I-p304 were low or similar to control mice. However, the IgG response to I-p2617 was significantly lower than in the controls. These results confirm the notion that the presence of I_2 atoms within p117 and p304 does not alter their antigenicity. However, the reduction of the IgG response to the Tg peptide I-p2617 in I_2-fed mice is more difficult to explain. If dietary I_2 antigenically modifies Tg, it might be possible that iodination of tyrosyls close to the I-p2617 site might alter the immunodominance hierarchy, resulting in a decreased response. If dietary I_2 does not antigenically modify Tg, it is hard to see why there is a selective IgG response to I-p2617, as was the case with the response to p2026 above.

Due to a lack of published data on the T cell involvement in IA-SAT in NOD.H2^{h4} mice, we decided to study IA-SAT at the T cell level. Iodine is incorporated into the Tg molecule (Dunn, 1995), but we do not know if Tg iodination level influences development of IA-SAT. We hypothesized that an increase in dietary I_2 intake by NOD.H2^{h4} mice will modify the Tg molecule generating neodeterminants. However, we could not detect T cell responses to any of the 18 Tg peptides, Tg, or HI-Tg in both spleen and cervical draining lymph nodes. This lack of response may be due to a variety of factors: 1) there may be a low frequency of Tg peptide-specific T cells present in the spleen and TDLN, 2) Tg-peptide specific T cells may be present in these areas, however, we have not tested the proper Tg peptides, and 3) T cell priming could have occurred earlier than 8 weeks and therefore the T cells may have migrated to the thyroid. As

previously mentioned, mononuclear cell infiltration has been recorded as early as 2 weeks after the addition of 0.05% NaI in the drinking water of NOD.H2^{h4} mice (Braley-Mullen *et al.*, 1999).

We tested three CD4^{+} T cell hybridomas specific for three iodinated Tg peptides. These cells were cultured in the presence of NOD.H2^{h4} BM-DC, which we used as APC. This experiment was conducted to ensure that the lack of detection of a T cell response in the spleen or lymph nodes was not due to a low frequency of effector T cells. Our results showed that none of the Tg peptides tested were generated after the processing of HI-Tg or Tg by NOD.H2^{h4} BM-DC. From this experiment we can conclude that the processing of HI-Tg in NOD.H2^{h4} APC does not generate the Tg peptides I-p117, I-p304, and I-p1931.

An increase in dietary I_2 intake did not enhance the Ab production to HI-Tg as compared to normal Tg or generate a T cell response to our panel of Tg peptides. This suggests that iodinated determinants in Tg may play a secondary role in the development of IA-SAT in NOD.H2^{h4} mice.

4.5 FUTURE DIRECTIONS

An important factor which should be examined is the I_2 content of Tg. Does Tg from NOD.H2^{h4} mice on 0.05% NaI water contain more I_2 than Tg from NOD.H2^{h4} mice on normal water? If it does, then I_2 may affect the immunogenicity or antigenicity in Tg directly and therefore, HI-Tg may play a role in the development of IA-SAT and SAT.

However, if it does not, then I_2 may have another role in the development of IA-SAT, such as effects on T_{regs}. Iodine may down-regulate T_{regs} which are important in inhibiting effector T cells which cause disease. Recent literature has shown that B cell-deficient NOD.H2^{h4} mice without T_{regs} develop severe SAT compared to B cell-deficient NOD.H2^{h4} mice which had their T_{regs} still intact (Yu *et al.*, 2006). A second study confirmed that T_{regs} play a role in IA-SAT development because the depletion of $CD4^+CD25^+$ T cells through the injection of anti-CD25 monoclonal Ab caused IA-SAT in NOD.H2^{h4} mice (Nagayama, *et al.*, 2007).

Another area which should be tested is the reactivity of thyroid-infiltrating T cells from NOD.H2^{h4} mice given NaI water for 8 weeks. T cells which have migrated to the thyroid may show T cell responses to our panel of Tg peptides in contrast to T cells isolated from the LN and spleen. The fact that there was extensive mononuclear cell infiltration in the thyroid glands of NOD.H2^{h4} mice after 8 weeks on NaI water shows that IA-SAT was induced and that there are mononuclear cells moving into the thyroid. Maybe the lack of a response to our panel of Tg peptides by T cells from the spleen and TDLN may be due to the Tg peptide-specific T cells migrating to the thyroid at a time point which is earlier than 8 weeks on NaI water.

Lastly, due to the high numbers of female control mice developing IA-SAT in our experiment it is very important that we know exactly when IA-SAT develops in our colony. Braley-Mullen *et al.* have documented the kinetics of SAT in the NOD.H2^{h4} mouse (Braley-Mullen *et al.*, 1999), however, the kinetics of SAT in our colony may not

be the same as the kinetics in her colony and this needs to be determined. With these new kinetics data we can better interpret our results and better plan our experiments in order to ensure optimum IA-SAT development in our mice on NaI water and normal water.

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