

STUDIES ON IODINE-ACCELERATED SPONTANEOUS
AUTOIMMUNE THYROIDITIS IN NOD.H2^{h4} MICE

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**STUDIES ON IODINE-ACCELERATED SPONTANEOUS AUTOIMMUNE
THYROIDITIS IN NOD.H2^{h4} MICE**

by

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LIST OF ABBREVIATIONS

a.a.	Amino acid
Ab	Antibody
APC	Antigen presenting cell
BB/W	BioBreeding/Worcester strain
BSA	Bovine serum albumin
BUF	Buffalo strain
CNBr	Cyanogen bromide
cpm	Counts per minute
CS	Cornell strain
DC	Dendritic cell
DCLN	Draining cervical lymph nodes
DIT	Diiodotyrosine
EAT	Experimental autoimmune thyroiditis
HI-Tg	Highly iodinated Tg
HLA	Human leukocyte antigen
HT	Hashimoto's thyroiditis
hTg	Human Tg
IDDM	Insulin-dependent diabetes mellitus
ISAT	Iodine-accelerated SAT
I-Tg	In vitro iodinated Tg

LI-Tg	Low iodine content Tg
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MIT	Monoiodotyrosine
mTg	Murine Tg
NA	Natural antibodies
NIS	Sodium iodide symporter
NOD	Non-obese strain
OS	Obese strain
OVA	Ovalbumin
PBS	Phosphate-buffered saline
SAT	Spontaneous autoimmune thyroiditis
SLE	Systemic lupus erythematosus
T3	Triiodothyronine
T4	Thyroxine
Tg	Thyroglobulin
TPO	Thyroid peroxidase
TNP-OVA	Trinitrophenyl-ovalbumin
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor

Chapter 1: Introduction

1.1. Hashimoto's thyroiditis

Hashimoto's thyroiditis (HT) or lymphocytic thyroiditis, also known as struma lymphomatosa, is the most common autoimmune disorder of the thyroid gland (Farwell et al., 1996) and was described by Dr. Hakaru Hashimoto in 1912 (Hashimoto, 1912). It is characterized by a spectrum of clinical features ranging from formation of germinal centers in the thyroid, fibrosis, thyroid cell hyperplasia and/or goitre formation usually leading to hypothyroidism (Weetman, 1996). Hallmarks of HT are also high titers of thyroid peroxidase (TPO)-specific and thyroglobulin (Tg)-specific antibodies in the sera of patients (Pearce et al., 2003; Weetman, 2004; Weetman et al., 1994). Antibodies specific for thyroid stimulating hormone receptor (TSH-R) have also been detected (Weetman et al., 1994).

The autoimmune basis of HT involves activation of thyroid antigen-specific B and T cells in lymph nodes (Pearce et al., 2003). A diversity of pathogenic mechanisms has been reported to contribute to the tissue injury in HT, but their individual relative importance remains unclear (Weetman et al., 1994). Thyroid-specific antibodies can participate in complement-mediated injury (Chiovato et al., 1993) and in antibody-dependent cellular cytotoxicity (Bogner et al., 1984) whereas TSH-R specific antibodies can block the interaction between thyroid stimulating hormone (TSH) and its receptor (Endo et al., 1978) and cause hypothyroidism. Thyroid injury can be mediated either by cytotoxic T cells or through the cytokines secreted by T cells (Weetman et al., 1994).

Susceptibility to HT is defined by genetic and environmental factors (Weetman, 2004). Primarily association of proteins of the human leukocyte antigen (HLA) like HLA-DR4 (Jenkins et al., 1992; Thompson et al., 1985), HLA-DR5 (Farid et al., 1981; Thomsen et al., 1983; Weissel et al., 1980) with HT has been reported. HLA-DR3 has also been associated with HT (Golden et al., 2005; Tandon et al., 1990) and HLA-DR3 transgenic mice develop severe thyroiditis after immunization with either murine or human Tg (Carayanniotis, 2007; Kong et al., 1996). Association of cytotoxic T-lymphocyte-Associated Antigen-4 with HT susceptibility remains controversial (Kotsa et al., 1997; Petrone et al., 2001). Environmental factors related to HT include iodine and selenium (Kohrle et al., 2005), pollutants like radioactive isotopes (Tronko et al., 2006), tobacco smoking (Vestergaard et al., 2002), polychlorinated biphenyls (Langer et al., 2007) and stress (Tsatsoulis, 2006), as reviewed recently (Duntas, 2008). Infections like *Yersinia enterocolitica* (Strieder et al., 2003) and with hepatitis C virus (Testa et al., 2006), as well as treatment of patients susceptible to HT with certain drugs like interferon- α and interferon- β have been also associated with HT (Schuppert et al., 1997).

1.2. Animal models for Hashimoto's thyroiditis

1.2.1. Experimental autoimmune thyroiditis

In 1956, experimental autoimmune thyroiditis (EAT) was demonstrated for the first time in rabbits by Rose (Rose et al., 1956). Rabbits immunized with thyroid extracts in complete Freund's adjuvant (CFA) were described to have undergone changes in their

thyroid glands and were reported to produce thyroid-specific antibodies in their sera. Similar observations were subsequently reported in mice (Rose et al., 1971), monkeys (Rose et al., 1966), dogs (Terplan et al., 1960), guinea pigs (Terplan et al., 1960), rats (Jones et al., 1961) and chickens (Jankovic, 1963). Most information regarding the cellular mechanisms and the genetics underlying EAT has been obtained in the murine model (Kong et al., 1994).

1.2.2. Spontaneous autoimmune thyroiditis

Spontaneous autoimmune thyroiditis (SAT) has been reported to develop in different animal species (Bigazzi et al., 1975; Ruwhof et al., 2001). Ninety percent of the Obese strain (OS) of White Leghorns chickens develop SAT leading to hypothyroidism vs 1% in their ancestor chickens of the Cornell-C (CS) strain of White Leghorns (Cole et al., 1968; Dietrich et al., 1999; Wick et al., 1974). Thyroid glands of OS chickens are characterized by the presence of lymphocytes and other mononuclear cells and Tg-specific antibodies can be detected in their sera (Sundick et al., 1979). SAT has also been described in BUF (Buffalo) rats (Hadju et al., 1969; Rose et al., 1976) as well as BioBreeding/Worcester (BB/W) rats, an animal model for insulin-dependent diabetes mellitus (IDDM) (Sternthal et al., 1981; Yanagisawa et al., 1986). Dogs developing SAT belong mainly in colonies of beagle (Beierwaltes et al., 1968; Bigazzi et al., 1975; Tucker, 1962). Marmoset monkeys have also been studied as an animal model for SAT (Bigazzi et al., 1975; Levy et al., 1972).

The non-obese diabetic (NOD) mouse is an animal model for IDDM (Anderson et al., 2005). NOD mice, expressing the K^d , $I-A^{g7}$, $I-E^{null}$, D^b major histocompatibility complex (MHC) haplotype, are characterized by infiltration of pancreatic islets with mononuclear cells (Many et al., 1996). Studies have also reported spontaneous mononuclear cell infiltration in 90% of the thyroid glands of NOD mice starting at 10 days of age while antibodies reacting with thyroid extracts were present in the sera of 42% of NOD mice that had developed SAT (Bernard et al., 1992). The incidence of SAT in NOD mice differs among colonies (Bernard et al., 1992; Damotte et al., 1997; Many et al., 1995; Many et al., 1996) while it is not associated with diabetes or insulinitis development suggesting that it is precipitated by a distinct mechanism (Bernard et al., 1992). High iodine intake leads to a significant increase in the incidence of SAT in NOD mice with $CD4^+$ and $CD8^+$ T cells required for optimal disease induction (Hutchings et al., 1999; Verma et al., 2000). Specifically, it was shown that mAb-mediated depletion of $CD4^+$ or $CD8^+$ T cells during the period of iodine administration led to decreased SAT incidence compared to control mice receiving only NaI-containing water for 6 weeks (Hutchings et al., 1999). Also, decreased SAT incidence was observed after adoptive transfers of $CD4^+$ or $CD8^+$ - depleted splenocytes to iodine-fed NOD-scid mice compared to iodine-fed NOD-scid mice receiving unseparated splenocytes (Verma et al., 2000). Another murine strain, NOD.H2^{h4}, generated from NOD (H-2^{g7}) and B10.A (4R) (H-2^{h4}) mice has been reported to develop SAT (Podolin et al., 1993). NOD.H2^{h4} mice express the H-2^{h4} MHC haplotype- K^k , $I-A^k$, $I-E^{null}$ and D^b alleles- while the rest of their genes are identical to the NOD genome. Fifty percent of NOD.H2^{h4} mice develop SAT vs 5% in the parental NOD strain (Burek et al., 2003). The high incidence of SAT in this strain probably results from

the combined presence of the A^k molecule which is permissive for thyroiditis development (Kong et al., 1997) and NOD non-MHC genes that are known to predispose to autoimmunity development. In support of this supposition, the parental B10.A(4R) (I-A^k) mice do not develop SAT and NOD (I-A^{g7}) mice are characterized by diabetes development (Weatherall et al., 1992; Wicker, 1997). Mononuclear cell infiltration in the NOD.H2^{h4} thyroids and Tg-specific serum IgG antibodies can be detected by 7-10 months of age (Braley-Mullen et al., 1999). NOD.H2^{h4} mice also develop periinsulinitis by 8-10 months of age but they are completely protected from diabetes development (Podolin et al., 1993).

1.3. Metabolism of iodine

Iodine is a trace element contained in various drugs, food preservatives, milk, eggs, seafood and vitamin preparations. Occasionally, bread made exclusively from seaweed or drinking water can be sources of iodine (Braverman, 1990; Roti et al., 2000). The main functions of iodine consist in thyroid hormone formation and regulation of thyroid function (Saller et al., 1998).

Dietary iodine enters the gastrointestinal tract where initially it is converted to inorganic iodide and then it is almost completely absorbed. Following absorption, inorganic iodide circulates between the plasma, the extracellular fluid, red blood cells and the thyroid gland. Some amount of iodide is found in the salivary glands, gastric mucosa, colon, mammary glands and the placenta. Sweat and breast milk also contain iodide. 95% of inorganic iodide is cleared via the kidneys (Cavalieri, 1997).

The major compartment storing iodine is the thyroid gland. Inorganic iodide is transferred to the thyroid via an active transport mechanism mediated by the sodium-iodide symporter (NIS) located in the basolateral membrane of the thyroid cells (Dai et al., 1996). Iodide moves along its electrochemical gradient to the apical surface of the cells where it is first oxidized by TPO in the presence of H_2O_2 . Subsequently, iodination of certain Tg tyrosyls and formation of thyroid hormone precursors, monoiodotyrosine (MIT) and diiodotyrosine (DIT) are catalyzed by TPO. Following this, certain tyrosyl residues donate their iodinated phenyl group to specific acceptor sites leading to thyroxine (T4) and triiodothyronine (T3) formation. The main acceptor sites are found at the 5, 1,291, 2,554 and 2,747 tyrosine residues in most species. The iodinated Tg enters the thyrocytes through pinocytosis and is directed to lysosomes where proteolysis occurs. MIT, DIT, T4 and T3 are released and iodotyrosine dehalogenase removes iodine from MIT and DIT for reuse within the thyroid or release into the blood (iodide leak) (Cavalieri, 1997; Dunn et al., 2009).

1.3.1. Iodine and its relation to thyroiditis development

According to the World Health Organization, the recommended daily allowance of iodine for adults amounts to 150 μg (World Health Organization, 2004). Low dietary supply of iodine has been related to goiter formation, mental retardation and cretinism in different areas of the world like Argentina, Switzerland, China and the United States. To increase the iodine intake, iodized oil or bread, milk, water and salt fortified with iodine were introduced in these countries (World Health Organization, 2004). Even though the

incidence of goiter and cretinism was decreased after iodine prophylaxis, an increase in Hashimoto's thyroiditis was reported relating the excess of dietary iodine to disease development (Harach et al., 1985; Hedinger, 1981; Rose et al., 1997; Rose et al., 2002; Rose et al., 1999; Sundick, 1990; Teng et al., 2008; Vagenakis et al., 1975; Weaver et al., 1966). Also, high iodine intake was shown to increase the incidence and/or severity of thyroiditis in animal models that develop SAT like CS chicken (Bagchi et al., 1985) and BB/W rats (Allen et al., 1986). Administration of excess iodine to hamsters with hyperplastic goiters resulted in thyroiditis development (Follis, 1959). The mechanism whereby iodine precipitates thyroiditis remains unknown.

1.3.2. Iodine-accelerated SAT in NOD.H2^{h4} mice

The role of iodine in thyroiditis development has been studied in NOD.H2^{h4} mice, an animal model for SAT. 6-8 week old NOD.H2^{h4} mice receiving water supplemented with 0.05% NaI (NaI H₂O group) for 8 weeks show accelerated incidence and severity of SAT (ISAT) compared to age-matched mice on plain water (control group) that show any sign of SAT. Also, Tg-specific IgG antibodies can be readily detected in the NaI H₂O group after 8 weeks of iodine administration (Braley-Mullen et al., 1999; Hutchings et al., 1999; Rasooly et al., 1996). Tg-specific IgG antibodies belong mainly in the IgG1 and IgG2b subclass as reported by Braley-Mullen et al. (Braley-Mullen et al., 1999). Other studies have described IgG2a, IgG2b and very low amounts of IgG1 (Rasooly et al., 1996) or very high titers of IgG1 in sera from few mice (Hutchings et al., 1999). IgM antibodies reacting with Tg have also been detected in the sera of iodine-fed and control mice

(Rasooly et al., 1996). In one study, the presence of Tg-reactive IgM has been reported but as shown binding to Tg appeared to be non specific (Braley-Mullen et al., 1999). TPO-specific antibodies were not detected in the sera of either group (Rasooly et al., 1996; Verma et al., 2000). The ISAT incidence is not higher in female mice compared to male mice (Rasooly et al., 1996) as opposed to the human disease where thyroiditis incidence is significantly higher in female compared to male individuals (Okayasu et al., 1994). Also, iodine administration in NOD.H2^{h4} mice was not found to alter T4 levels in the serum (Rasooly et al., 1996; Teng et al., 2009) as was previously described in SJL mice (Li et al., 2007).

ISAT characteristics are unique among transgenic and congenic NOD MHC strains. NOD.SWR (H-2^q MHC haplotype) mice placed on NaI-H₂O for 8 weeks did not develop either thyroid lesions or Tg-specific IgG antibodies (Braley-Mullen et al., 1999). Iodine administration for 6 weeks in 3 month-old NOD and transgenic NOD mice-expressing an I-E α^d or a mutated I-A β^{g7} chain, - increased the incidence of ISAT but, the percentage of affected animals was significantly lower compared to NOD.H2^{h4} mice. Also, none of the NOD and transgenic NOD strains developed Tg-specific IgG antibodies (Hutchings et al., 1999; Verma et al., 2000) indicating that the NOD.H2^{h4} genetic background confers high susceptibility to ISAT development compared to other congenic NOD MHC environments.

So far, studies have highlighted the involvement of different T cell subsets in ISAT development. Immunohistochemical data have shown that the first type of cells

infiltrating the thyroid gland after 2 weeks of iodine administration are CD4⁺ and CD8⁺ T cells (Bonita et al., 2003; Verma et al., 2000; Yu et al., 2001). Depletion of CD4⁺ and/or CD8⁺ T cells in NOD.H2^{h4} mice after 2-3 weeks of iodine administration results in minimal or no thyroiditis and reduction of Tg-specific IgG antibodies indicating their important role in disease progression (Braley-Mullen et al., 1999; Hutchings et al., 1999; Verma et al., 2000). Another T cell subset important for ISAT is CD4⁺CD25⁺ cells. Depletion of this cell type in NOD.H2^{h4} mice before and throughout iodine administration using an anti-CD25 monoclonal antibody (mAb) for 8 weeks resulted in increased severity of thyroiditis (Nagayama et al., 2007). Similar results were reported after depleting CD4⁺CD25⁺ cells in NOD.H2^{h4} B cell-deficient mice beginning at the age of 10 days (Yu et al., 2006). Studies have also highlighted that NKT cells may be involved in thyroiditis pathogenesis (Burek et al., 2003). Tg-specific T cell lines generated from spleens of iodine-fed NOD.H2^{h4} mice expressed CD3⁺CD4⁺DX5⁺, markers for both T and NK cells as well as the V α 14J α 281 gene rearrangement, characteristic of NKT cells (Sharma et al., 2002). Adoptive transfers of these T cell lines to NOD.H2^{h4} recipients led to increased thyroid infiltration and enhanced Tg-specific Ab production (Burek et al., 2003).

B cells play a major role in thyroiditis development in NOD.H2^{h4} mice (Braley-Mullen et al., 2000; Yu et al., 2008; Yu et al., 2006). Depletion of B cells using anti-IgM mAb during the period of iodine administration in NOD.H2^{h4} mice resulted in minimal or no thyroiditis development compared to control mice injected with saline that developed ISAT (Braley-Mullen et al., 2000). Similar observations were made when B cells were

depleted using a chimeric mouse human anti-CD20 mAb, called rituximab, (Yu et al., 2008) that recognizes murine CD20 but can also bind human C1q and mediates antibody-dependent cellular cytotoxicity with human effector cells (Reff et al., 1994). Also, minimal or no ISAT was observed in iodine-fed NOD.H2^{h4} B cell deficient mice-called NOD.K μ ^{null}, compared to iodine-fed NOD.H2^{h4} mice that developed thyroiditis (Braley-Mullen et al., 2000). B-cell depletion studies showed that the presence of B cells in ISAT is crucial in the first weeks after birth and they may have a role in T cell maturation. It was shown that T cells developed in B cell presence were able to induce ISAT after transfer in lethally irradiated NOD.K μ ^{null} mice whereas T cells from B cell-deficient mice did not induce thyroiditis after transfer in NOD.K μ ^{null} mice. B cells may be involved in ISAT development as antigen-presenting cells (APC) since passive transfer of NOD.H2^{h4} sera to NOD.H2^{h4} B cell deficient mice for 5 weeks starting 3 weeks after iodine administration did not cause disease indicating that autoantibody production has a minor role in disease development (Braley-Mullen et al., 2000).

In addition to B cells, a study has shown that maturation of NOD.H2^{h4} dendritic cells (DC) is defective in vitro (Strid et al., 2001). Bone-marrow derived DC generated in the presence of GM-CSF failed to upregulate I-A and CD86 expression upon stimulation with LPS/IFN- γ for 18 hours indicating that there is a defect in the NOD.H2^{h4} genome affecting the differentiation of DC. In the same study, the inability of NOD.H2^{h4} DC to stimulate a I-A^k- restricted, hen egg lysozyme peptide (46-61)-specific T cell hybridoma was interpreted to be due to low expression of MHC class II and CD86, whereas peptide pulsed CBA/J-derived DC were able to activate the same T cell hybridoma.

Certain cytokines participate in the regulation of ISAT. It was shown that depletion of TGF- β for 4 weeks after the onset of iodine administration leads to low incidence and severity of ISAT as well as reduction in Tg-specific IgG antibody production via an unknown mechanism (Braley-Mullen et al., 2001). In contrast, IL-4 deficient mice developed ISAT to the same extent as wild type mice after 8 weeks of iodine intake (Yu et al., 2002) indicating that IL-4 does not play a key role in ISAT. Interestingly, in the same study IFN- γ deficient mice did not develop typical thyroid lesions after iodine administration but thyroid epithelial cell enlargement and few lymphocytes were observed (Fang et al., 2007; Yu et al., 2002). These data were interpreted to mean that IFN- γ is required for ISAT progression but inhibits thyrocyte proliferation. In a different experimental system, transgenic NOD.H2^{h4} mice overexpressing IFN- γ in the thyroid developed thyroiditis at a lower incidence and severity than wild-type littermates after immunization with Tg in CFA indicating that IFN- γ can restrict thyroiditis development (Barin et al., 2003). Also, the role of IFN- α in ISAT has been examined since IFN- α treatment is known to induce thyroiditis in up to 40% of patients with carcinoid tumors and hepatitis C. NOD.H2^{h4} mice on water with 0.05% NaI for 8 weeks were also given anti-IFN α mAb but ISAT incidence and severity as well as production of Tg-specific IgG in the sera were not affected compared to mice given saline (Oppenheim et al., 2003).

1.3.3. Effects of iodine on Tg

The iodine content of Tg has been shown to affect its immunogenicity/antigenicity at the T level (Carayanniotis, 2007). As shown by Barin et al., a Tg-specific CD4⁺ T cell line,

2D11, generated from splenocytes of iodine-fed NOD.H2^{h4} mice could not respond to Tg with low iodine content (LI-Tg) while proliferation to Tg with normal iodine content was recorded indicating that Tg iodine content was critical for T cell recognition (Barin et al., 2005). Similar observations were made for two T cell hybridomas generated after fusion of CBA/J Tg-specific T cell lines with BW5147 cells. These hybridomas were stimulated by human (h) or murine (m) Tg containing 3-4 T4 per molecule, whereas very poorly iodinated hTg or mTg failed to activate either of the hybridomas (Champion et al., 1987). At the polyclonal level, higher T cell responses against in vitro highly iodinated Tg (HI-Tg), containing >120 I atoms per molecule, versus Tg with normal iodine content were reported using lymph node cells (LNC) from HI-Tg/Tg challenged SJL mice (Dai et al., 2002).

Another important parameter affecting Tg recognition by T cells is the presence of iodine in certain tyrosyl residues (Carayanniotis, 2007). It has been shown that the presence of iodotyrosyls at 130, 306 and 1942 residues of Tg led to the formation of neoantigenic T cell epitopes which also elicited EAT in CBA/J mice whereas their non-iodinated analogs were not immunogenic (Li et al., 2006). Also, iodotyrosyl formation was shown to have modifying effects on the immunogenicity of distinct Tg peptides since proliferative responses of LNC from CBA/J mice primed with iodinated/non-iodinated peptide analogs were increased, suppressed or not altered in the presence of iodinated vs non-iodinated forms of these peptides (Li et al., 2007). Variable effects of iodine on the T cell recognition have been also reported at the clonal level. For example, incorporation of an iodine atom to tyrosyl 192 in Tg peptide p179 did not affect the activation of some p179-

specific or I-p179-specific T cell hybridomas whereas the activation of other p179-specific or I-p179-specific T cell hybridomas was abolished or increased (Jiang et al., 2007).

At the B cell level, Sundick et al., were the first group to describe that normal K strain chickens immunized with highly iodinated Tg (HI-Tg, 60-145 I atoms per Tg molecule) produced antibodies to HI-Tg, T3 and T4 compared to LI-Tg-challenged chickens that produced very low levels of antibodies to LI-Tg, T3 and T4 (Sundick et al., 1987). Also, immunization of NB rats - a subline of BB/W rats - with Tg of normal iodine content elicited higher production of Tg-specific antibodies compared to rats immunized with LI-Tg (Ebner et al., 1992). Studies from Burek's laboratory have shown that iodine can affect the formation of epitopes recognized by B cells. Specifically, a panel of murine mAbs was generated against hTg containing 19 atoms of iodine per Tg molecule. MAb reactivity was tested against hTg, Tg with no detectable iodine and in vitro iodinated Tg containing 150 atoms per Tg molecule. The data showed that some mAbs failed to react to Tg devoid of iodine whereas other mAbs failed to react to HI-Tg indicating that epitopes recognized by B cells were modified by the presence or the absence of iodine (Saboori et al., 1998a; Saboori et al., 1998b). This group has also reported that antibodies derived from NOD.H2^{h4} mice on NaI/normal water recognized preferentially Tg with a physiological iodine content relative to LI-Tg (Barin et al., 2005).

1.3.4. Effects of iodine on thyrocytes and immune cells

Excessive iodine administration has been known to induce necrosis of thyrocytes in autoimmune-prone animals. Thyroid cells from BB/W rats treated with moderate (3×10^{-6} M) or high (3×10^{-3} M) iodine doses for 12 weeks underwent ultrastructural changes compared to thyrocytes from rats on water without NaI (Li et al., 1994). Those thyrocytes were characterized by accumulation of secondary lysosomes and lipid droplets, swollen mitochondria and dilatation of rough endoplasmic reticulum under electron microscopy. Nuclear changes were observed including condensation of nuclear chromatin, nuclear disintegration (karyolysis) and nuclear fragmentation (karyorrhexis), indications of cell necrosis. Thyrocytes from control rats were morphologically intact under electron microscopy whereas some were characterized by mild dilatation of rough endoplasmic reticulum and occasional mitochondrial swelling (Li et al., 1994). Similar characteristics were described in thyroid cells from NOD.H2^{h4} mice fed with various iodine doses ranging from 1×10^{-5} – 2×10^{-3} M for 8 to 24 weeks. The observed changes were iodine dose-dependent and they were more distinctive as the duration of iodine administration was expanded (Teng et al., 2009). Likewise, thyrocytes from OS chickens administered 250 µg of iodine intraperitoneally had swollen mitochondria and rough endoplasmic reticulum whereas in the nucleus the chromatin was clumped 12 hours after iodine administration (Bagchi et al., 1995). Similar observations have been reported in iodine-deficient goitrous mice (Mahmoud et al., 1986; Many et al., 1995) and hamsters with hyperplastic goiters (Follis, 1959). Human thyroid follicles cultured in vitro with high doses of iodine (10^{-3} M) were reported to develop signs of cellular necrosis (Many et al., 1992). Other published iodine effects on thyrocytes include upregulation of intracellular

adhesion molecule-1 expression through a reactive oxygen species-dependent pathway in NOD.H2^{h4} thyrocytes (Sharma et al., 2008) and 50 % reduction of TSHR, NIS, Tg and TPO mRNA expression in the rat thyroid follicular cell line PCC13 (Leoni et al., 2008).

Since iodine has been suggested to increase thyroiditis incidence and autoantibody synthesis (Rose et al., 1999), studies have examined whether iodine has stimulating effects on B cells. Human peripheral blood lymphocytes were stimulated with pokeweed mitogen and were cultured in the presence of 10^{-6} to 10^{-3} M potassium iodide or potassium chloride. It was shown that, in the presence of iodide, the number of cells synthesizing IgG as well as the amount of IgG released in the supernatant was significantly increased compared to lymphocytes cultured with potassium chloride indicating that iodine can enhance antibody synthesis (Weetman et al., 1983). Another effect of iodine on immune cells is the enhanced ability of monocytes to mature into DC after culture with iodinated compounds. Human peripheral blood monocytes were cultured overnight in the presence of T3 or T4 or Tg with high (0.37% w/v), low (0.2%) or no iodine (0%) content in concentrations ranging from 2×10^{-18} M to 2×10^{-9} M. It was shown that the number of DC generated from monocytes was increased after culture with iodinated compounds and thyroid hormones compared to spontaneous monocyte to DC transition or after incubation with thyronine- deiodinated form of T4. The data from this study suggest that iodinated compounds and thyroid hormones can promote the maturation of human peripheral blood monocytes to DC (Mooij et al., 1994).

1.4. Natural antibodies

Antibodies reactive with self or foreign antigens are present in the sera of normal individuals in the absence of antigenic stimulation (Coutinho et al., 1995; Lacroix-Desmazes et al., 1998; Quintana et al., 2004). They are designated as natural antibodies (NA) and they have been detected in the sera of humans (Berneman et al., 1993; Guilbert et al., 1982), mice (Berneman et al., 1992), rabbits, rats (Avrameas, 1991) and various fish species (Adelman et al., 2004; Gonzalez et al., 1988). NA are preferentially but not exclusively, produced by B-1 cells (Avrameas et al., 2007). Most of them are of the IgM and IgG isotype but IgA can also be found in the blood, colostrum, saliva and cerebrospinal fluids (Avrameas et al., 2007). NA possess some characteristic properties differentiating them from conventional antibodies. The majority of NA are polyreactive recognizing several evolutionarily conserved antigens like insulin, Tg as well as intracellular constituents including actin, tubulin, DNA, myelin basic protein and other proteins (Avrameas, 1991). NA binding to antigens have low affinity compared to monoreactive antibody binding (Avrameas et al., 2007; Notkins, 2004) and most NA are encoded by non-mutated germline genes (Avrameas et al., 2007; Lacroix-Desmazes et al., 1998; Zelenay et al., 2007).

The biological functions attributed to NA are participation in the clearance of apoptotic cells (Peng et al., 2005), or in oxidative stress of erythrocytes via recognition of self-antigens such as human red blood cell band 3 protein (Lutz et al., 1987). Also, elimination of cancer cells after NA recognition of carbohydrate groups such as Lewis^x, also designated CD174, and glycoproteins such as mucin 1, has been reported (Vollmers

et al., 2007). NA able to recognize IL-1 α , TNF- α , interferon- α and interferon- β have been recorded in the sera of normal adults and it has been proposed that such NA can carry these cytokines in the circulation inhibiting proteolytic degradation and preventing their urinary excretion and absorption to vessel walls (Bendtzen et al., 1990). NA can regulate immune responses mediated by autoreactive T cells since normal human serum can suppress the proliferative response in an autologous mixed lymphocyte reaction (Wolf-Levin et al., 1993). Murine IgM NA are also known to regulate the binding of IgG autoantibodies to self antigens through an idiotypic interaction. Specifically, it was shown that BALB/c, B/W and BALB/B6-derived serum IgG reactivity to actin, DNA, TNP and tubulin was low whereas purified IgG reactivity against the same panel of antigens was significantly higher. It was shown that the presence of IgM could inhibit IgG binding activity to antigens because IgM was able to interact with F(ab')₂ fragments of IgG indicating that NA are organized in an idiotypic-like network (Adib et al., 1990). NA also participate with complement in hyperacute rejection observed in xenotransplantation of vascularized grafts (Platt et al., 1990). Other NA functions involve elimination of pathogenic agents (Ochsenbein et al., 2000), penetration into cells (Ruiz-Arguelles et al., 2003) and expression of enzymatic activities (Paul et al., 1990).

1.4.1. NA in autoimmunity

According to the concept of the “immunological homunculus”, natural T and B cell autoimmunity is directed against a particular set of self antigens and dysregulation of natural autoimmunity leads to development of autoimmune disorders (Cohen, 2000). This concept is supported by data describing that pathologic autoantibodies in autoimmune

diseases might emerge from a pool of NA (Quintana et al., 2004). NA and autoantibodies present in autoimmune diseases, especially in systemic lupus erythematosus (SLE), are indistinguishable in terms of V gene usage and polyreactivity (Avrameas, 1991; Quintana et al., 2004) but the relationship between the two Ab categories remains unclear. Generation of B cell hybridomas from newborn to 12 month old B/W mice developing SLE has shown that there was a selective maintenance of DNA-specific polyreactive IgM Ab and an increase in DNA-specific polyreactive and monoreactive IgG Ab production during disease development. The overall percentage of autoreactive antibodies remained steady throughout these 12 months indicating a shift of NA toward pathogenic IgG autoantibody production (Gilbert et al., 1992). Also, DNA-specific polyreactive IgG has been detected in the sera of patients with SLE (Matsiota et al., 1987). Insulin-specific IgG mAb generated from splenocytes of diabetic NOD mice have been shown to respond with low avidity to insulin and to react with other self antigens like Tg and DNA. Their CDR3 regions were characterized by limited N segment diversity (Thomas et al., 2002). Insulin-specific IgG able to react with DNA and Tg have also been described in patients with type I diabetes (Potter et al., 2000).

1.5. Thesis aims

High iodine intake in NOD.H2^{h4} mice accelerates SAT development as well as production of Tg-specific IgG via an unknown mechanism. The goals of this study are: a) to examine whether excess iodine precipitates ISAT development via the formation of Tg with high iodine content in vivo. Increased iodine incorporation in Tg may lead to formation of pathogenic epitopes not generated in Tg. Also, it will be tested whether differential reactivity against Tg and I-Tg preparations at the B and T cell level can be detected. Higher responses to I-Tg vs Tg at the B and/or T cell level would provide indirect evidence for the formation of I-Tg in vivo (Chapter 3); b) to investigate whether Tg-binding IgG, that is produced without T cell help since very low or undetectable T cell responses to Tg have been reported, belong to the NA repertoire. In iodine-fed NOD.H2^{h4} mice polyreactivity of Tg-binding IgG will be examined at the serum level, at the purified IgG level and following purification of Tg-binding IgG against Tg, I-Tg, actin, DNA and TNP-OVA (Chapter 4).

Chapter 2: Materials and Methods

2.1. Animals

NOD.H2^{h4} mice, originally derived by Dr. L. Wicker (Merck Laboratories, Rahway, NJ), were kindly provided to us by Dr. H. Braley-Mullen (University of Missouri, Columbia, MO). CBA/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were bred and maintained under specific pathogen-free conditions in the animal facility of the Faculty of Medicine, Memorial University of Newfoundland. Both male and female mice were used and they were age- and sex-matched in each experiment. Mice received 0.05% NaI in their drinking water beginning at 6-8 weeks of age and thyroids or sera were obtained at various time intervals.

2.2. Purification of Tg

Frozen thyroids of outbred BALB/c mice (Bioproducts for Science, Indianapolis, IN) were homogenized in PBS buffer, pH=7, containing 10^{-6} M leupeptin, 10^{-6} M pepstatin and 10^{-3} M phenylmethanesulfonylfluoride (Sigma). Thyroid extracts were centrifuged 3x at 16,000 g for 10 minutes at 4°C, and supernatant subsequently was passed through a Sepharose CL-4B column (Amersham Biosciences, Sweden). Collection of fractions was performed using a microfraction collector (Gilson model 203, Mandel Scientific) and the optical density of fractions was determined at $\lambda=280$ (Life Science UV/Vis spectrophotometer, Beckman Coulter, Du[®] 730). The fractions forming the second peak of the chromatogram were pooled, dialyzed 3x in phosphate-buffered saline (PBS), pH=7, concentrated using the Amicon Filter (Millipore Corporation, Bedford, USA) to 1 mg/ml and stored at -20° C.

2.3. Tg iodination and determination of I content in Tg

Tg iodination was conducted using the Iodo-Beads Iodination Reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, one bead was added to aliquots containing 200 μ l PBS and 20 μ l of 2.5 mM NaI for 5 min, followed by the addition of 200 μ g of Tg for 30 seconds. The beads were then removed and the samples were pooled and dialyzed 3X in HPLC-grade water. This protocol generated mildly iodinated I-Tg, containing approximately 24 I atoms/monomeric subunit vs 14 I atoms in control Tg. A non incinerative method based on the catalytic activity of iodine in the ceric-arsenite reaction was used to determine Tg iodine levels, as previously described (Dai et al., 2002). The reduction of ceric ion (Ce (IV) to cerious ion (Ce (III) by arsenic (As) leads to the conversion of the yellow ceric ion to the colorless cerious ion, a reaction that is followed spectrophotometrically at $\lambda=410$ nm. Standard curves were constructed using known concentrations of T4 (Sigma) dissolved in 99 vol methanol and 1 vol 30% ammonium hydroxide. The iodine content of each Tg sample was taken as the average value of two to three independent measurements.

2.4. Measurement of IgG and IgM responses by ELISA

IgG and IgM responses were detected by ELISA assay. Briefly, wells of polyvinylchloride plates were coated with 100 μ l of 10 μ g/ml Tg, I-Tg, actin (Sigma) and TNP-ovalbumin (Biosearch Technologies Inc.) in carbonate buffer pH=9.6. For DNA coating, wells were treated with 100 μ l of 0.01% solution of poly-L-lysine (Sigma) for 1 hour at room temperature. Then the plate was washed 3x with double distilled water and 100 μ l of 2.5 μ g/ml of DNA in borate-buffered saline, pH=8.4 were added in each well.

To confirm that actin, TNP-ovalbumin and DNA coating was efficient, preliminary experiments using monoclonal Abs against actin (IgG2a) (Sigma), TNP (IgG1) (BD Biosciences) and DNA (IgG2b) (Mybiosource.com) were performed (data not shown). After overnight incubation at 4°C, the plate was washed with PBS and blocked with 0.1% BSA in PBS for 1 hour at room temperature. The plates were again washed and serum samples or purified Tg-reactive IgG diluted in PBS/Tween 20 containing 0.1% BSA, were added for 1 hour. An alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc) and an alkaline-phosphatase-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch, Inc) were used to detect the presence of bound antibodies. After 1 h incubation, the p-nitrophenyl phosphate substrate (Sigma) was added to the plates and light absorption was determined at 405 nm using a Vmax plate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as mean O.D. values of triplicate wells \pm S.D. after subtraction of background values from wells with no serum added. In competitive inhibition assays, the binding of serum IgG (50 μ l serum at 1:200 dilution per well) to Tg or I-Tg was monitored in the presence of increasing amounts of soluble inhibitor. Percent inhibition was determined by the formula: $[1 - (\text{experim. O.D.} / \text{max. O.D.})] \times 100$ and was expressed as a mean of triplicate wells \pm S.D.

2.5. [³H]-thymidine incorporation assay

At various time intervals following administration of NaI in the drinking water, cervical lymph node or spleen cell suspensions were prepared in HL-1 medium (Lonza Walkersville, MD, USA) supplemented with 100 U/ml penicillin/streptomycin (Gibco,

Invitrogen Corporation), 2 mM glutamine (Gibco, Invitrogen Corporation) and 50 μ M 2- β -mercaptoethanol (Sigma). Cells, adjusted to 4×10^6 cells/ml, were cultured in the presence of titrated amounts of Tg or I-Tg for 72 hours. During the last 18 hours of culture, 1 μ Ci [3 H]-thymidine (Perkin Elmer, Boston, USA) was added into each well. The cells were then harvested on a ClassicCell Harvester (Skatron Instruments, Lier, Norway) and radioactivity was measured in a Beckman Coulter LS6500 scintillation counter. Stimulation index is defined as counts per minute in the presence of antigen/counts per minute in the absence of antigen.

2.6. Histological assessment of thyroid infiltration by mononuclear cells

Thyroids were aseptically removed, fixed in 10% formalin, embedded in methacrylate and step-sectioned in 4 μ m thick sections as previously described (Rao et al., 1999). Staining with hematoxylin and eosin followed and the presence of mononuclear cell infiltration was scored as follows: 0: no infiltration, 1: interstitial accumulation of cells between two or more follicles, 2: one or two foci of cells at least the size of one follicle, 3: diffuse infiltration of 10- 40% of the total area, 4: extensive infiltration of 40-80% of the total area, 5: extensive infiltration of more than 80% of the total area. Results report the highest infiltration index observed among the step sections of each gland.

2.7. Purification of IgG from mouse serum using a Protein-G-Sepharose 4 column

A Protein-G-Sepharose 4 Fast Flow (GE Healthcare) column was equilibrated using 100 ml of 0.05 M phosphate, 0.15 M NaCl, pH=7 as a binding buffer. An equal volume of binding buffer was added to the serum volume and then loaded to the column running

with binding buffer. Fractions were collected and O.D. readings at $\lambda=280$ nm were determined to verify that background levels in O.D. values were reached. Then IgG antibodies were eluted from the column using 0.05 M glycine-hydrochloride, 0.15 M NaCl, pH=2.5, as an elution buffer. The IgG-containing fractions were pooled and neutralized using 100 μ l of 1 M Tris-HCl, pH=9.0, per ml of pooled eluate. The IgG-containing fractions were dialyzed 3x in PBS, pH 7.0, concentrated to the initial volume of the loaded serum sample, and the yield was assessed as mg IgG/ml serum. 0.05% NaN₃ was added to IgG samples stored at 4° C.

2.8. Coupling Tg to CNBr-activated Sepharose 4B

CNBr-activated Sepharose 4B (Sigma) was washed and swollen using 200 ml of cold 1 mM HCl for 30 minutes. The supernatant containing lactose was removed by gentle suction in a Büchner funnel and the resin was washed with 40-80 ml of distilled water. Following the resin was washed with 5 ml of 0.1 M NaHCO₃ buffer containing 0.5 M NaCl, pH=8.3-8.5 (coupling buffer) and immediately was transferred to solution containing 10 mg of Tg in coupling buffer. The protein and the resin were mixed for 2 hours using an end-over-end mixer and the resin was then washed with 40-80 ml of coupling buffer. To block unreacted groups of the resin, 0.2 M glycine, pH=8.0 (blocking solution) was added to the resin for 2 hours at room temperature. Then a cycle of 40-80 ml of coupling buffer followed by 40-80 ml of 0.1 M acetate buffer containing 0.5 M NaCl, pH=4 wash was repeated 5x to wash the resin off the blocking solution. The resin was equilibrated using 20 ml of PBS, pH=7.0 containing 0.05% NaN₃ and was stored at 4°C.

2.9. Purification of Tg-specific IgG using a Tg-coupled CNBr-activated Sepharose

4B packed column

Tg-coupled CNBr-activated Sepharose 4B (Pierce, Rockford, IL, USA) was added to a 5 ml centrifuge column. The column was equilibrated using 20 ml of 0.05 M phosphate, 0.15 M NaCl, pH=7.0 as a binding buffer. The IgG sample was added to the column, let stand for 15 minutes and the unbound IgG was collected. After washing the column with 20 ml of binding buffer the optical density of the eluate was monitored until it returned to baseline. Then, Tg-specific IgG was eluted using 5 ml of 1 M Tris solution, pH=10.5. Tg-specific IgG fractions were pooled and were transferred to microconcentrator Centricon YM-30 (Centricon Centrifugal Filter Devices, Millipore) to change the solvent from Tris solution, pH=10.5 to PBS, pH=7.0 adding 500 μ l PBS 5x. Tg-specific IgG fractions were concentrated to the initial volume of the serum sample and stored at 4°C after adding 0.05% NaN₃.

2.10. Statistical analysis

Statistical comparison of SAT scores was performed by the non parametric Mann-Whitney test, whereas differences in iodine content between Tg preparations were assessed by the Student's unpaired t test, using GraphPad Prism 4.0 software (GraphPad Inc., San Diego, CA).

Chapter 3: Lack of association between iodine-accelerated thyroiditis and iodination levels of thyroglobulin in NOD.H2^{h4} mice

3.1. Abstract

High iodine intake has been reported to increase the incidence and severity of SAT in NOD.H2^{h4} mice but the mechanism underlying this phenomenon remains unknown. In this study, we examined whether this dietary regimen could precipitate SAT via an increase in the iodine content of Tg, a key autoantigen in thyroid disease. Because alterations were found in the chromatographic profile of Tg isolated from NOD.H2^{h4} mice, we assessed the iodine content of Tg from B-cell deficient NOD.H2^{h4} mice, (NOD.K μ ^{null}), which do not develop SAT. The iodine content of Tg from control mice (9.0 ± 2.7 I atoms per monomeric subunit) was not significantly different from that of Tg derived from mice placed on water containing 0.05% NaI for 4 weeks (10.9 ± 0.3 I atoms per monomer), indicating that in the NOD.H2^{h4} environment, excess iodine intake does not affect the iodination levels of Tg. Furthermore, in iodine-fed NOD.H2^{h4} mice, strong but equivalent serum IgG responses were detected to both Tg or in vitro iodinated Tg (I-Tg), suggesting lack of differential immune responses to putative neoantigenic Tg determinants modified in vivo by excess iodine. Similarly, thyroid-draining cervical lymph node cells or splenocytes from iodine-fed NOD.H2^{h4} mice responded weakly but equally well to Tg or I-Tg in vitro. These results demonstrate that iodine-accelerated SAT in NOD.H2^{h4} mice does not associate with a high Tg iodine content or differential B- or T-cell responses to I-Tg. Instead, the data suggest that dietary iodine may amplify immune dysregulation in this strain.

3.2. Introduction

The interplay between genetic predisposition and environment underlying the induction of autoimmune disease has been clearly shown in NOD.H2^{h4} mice, a model of SAT. This congenic strain, expressing the K^k,A^k, E⁰,D^b molecules in the NOD background, develops periinsulinitis but not diabetes (Podolin et al., 1993) and exhibits SAT at a higher incidence (50%-70% vs 5%) than the NOD strain (Braley-Mullen et al., 1999; Burek et al., 2003). Addition of NaI to the drinking water accelerates the onset of SAT from 7-10 months in control mice to 2 months after the initiation of the dietary regimen in NOD.H2^{h4} hosts (Braley-Mullen et al., 1999) and significantly enhances both its incidence and severity (Hutchings et al., 1999; Rasooly et al., 1996). Increased iodine ingestion has previously shown similar effects in other animal models of SAT such as the CS chicken (Bagchi et al., 1985) and the BB/w rat (Allen et al., 1986), but the NOD.H2^{h4} model has attracted attention in recent years due to the availability of well characterized mouse biomarkers and reagents. Epidemiological data clearly support the view that an iodine-rich diet can also promote subclinical hypothyroidism in humans (Bournaud et al., 2003; Konno et al., 1994; Markou et al., 2001), but the pathogenetic mechanisms remain mostly unknown.

Tg, the only protein with a capacity to store available iodine, is a natural focus of research on ISAT for several reasons. First, the titers of Tg-specific IgG1 and IgG2b Abs correlate with the ISAT severity (Braley-Mullen et al., 1999; Rasooly et al., 1996) suggesting that Tg is an early autoimmune target in the disease cascade. Second, the iodine content of Tg has been shown to correlate with its immunopathogenicity in studies on Tg-induced EAT (Champion et al., 1987; Dai et al., 2002; Sundick et al., 1987), and

with its antigenicity in studies of EAT (Saboori et al., 1998a; Saboori et al., 1998b) and ISAT (Barin et al., 2005). Third, we have previously provided evidence that iodination may alter the immunodominance hierarchy of pathogenic T-cell epitopes within Tg (Dai et al., 2002), and that iodotyrosyl formation within certain Tg peptides may create thyroiditogenic T cell epitopes (Li et al., 2006). Lastly, formation of Tg with a high iodine content has been observed in CS chickens placed on iodine-rich drinking water (Sundick et al., 1987) but this has not been confirmed in other species. In fact, we have recently shown that consumption of increased amounts of iodine precipitates hypothyroidism in SJL mice with no apparent autoimmune basis (Li et al., 2007).

In this report, we have examined whether the genetic background of NOD.H2^{h4} mice favors formation of I-Tg when the animals follow an iodine-rich diet. We have also tested whether NOD.H2^{h4} mice developing ISAT generate IgG responses that preferentially recognize I-Tg vs Tg, a finding that would indirectly support formation of I-Tg in the disease process. Lastly, we have tested T-cell responses of NOD.H2^{h4} mice with ISAT against Tg and I-Tg. Our results do not support the view that ISAT in NOD-H2^{h4} mice is associated with I-Tg formation or a response to neoantigenic Tg determinants containing iodine. Instead, they suggest that iodine may accelerate disease by amplifying dysregulation at other levels of the immune response.

3.3. Results

3.3.1. *The NOD.H2^{h4} genetic environment does not predispose for increased iodine incorporation in Tg*

To investigate whether elevated iodide intake leads to enhanced iodine incorporation in Tg, six-week-old NOD.H2^{h4} mice (n=13) were placed on drinking water supplemented with 0.05% NaI for 4 weeks, whereas age-matched control NOD.H2^{h4} mice (n= 13) received normal drinking water for the same period. Tg was subsequently isolated from pooled thyroids by gel filtration. Since the mononuclear cell infiltration of thyroids in ISAT peaks after 8 weeks of iodide supplementation (Braley-Mullen et al., 1999) it was reasoned that the thyroids of either group would yield sufficient amounts of Tg within the 4-week observation period. Surprisingly, it was found that the chromatographic elution profile of Tg from NOD.H2^{h4} mice did not show a 660 kDa soluble Tg peak, regardless of the dietary regimen used (**Fig. 1, A & B**). In contrast, Tg extracted from the thyroids of CBA/J mice (n=19) placed on iodide-supplemented water or BALB/c mice (n=13) on normal water was eluted in a distinct peak (**Fig. 1, C & D**). To investigate whether this serendipitous finding was likely to reflect the emerging Tg-specific autoreactivity in NOD.H2^{h4} hosts, we made use of NOD.K μ ^{null} mice that share all the MHC and non-MHC genes of the NOD.H2^{h4} strain but lack B cells due to the deletion of the I μ gene and do not develop ISAT (Braley-Mullen et al., 2000). We observed that Tg extracted from NOD.K μ ^{null} mice (n=13) with or without iodide supplementation, had a normal elution profile (**Fig. 1, E & F**). Iodine determination assays, based on a non incinerative method and making use of thyroxine standard curves (**Fig. 2A**) showed that Tg from NOD.K μ ^{null}

mice on NaI water contained 10.9 ± 0.3 I atoms per monomer which was not statistically different from the I content of Tg from control NOD.K μ^{null} mice (9 ± 2.7 I atoms per monomer) (**Fig. 2B**). These values were in accordance with the I content of Tg from BALB/c mice on normal water (13.6 ± 1.5 I atoms per monomer), or Tg from CBA/J mice on NaI/water (8.7 ± 1.0 iodine atoms per monomer) (**Fig. 2B**). These results suggested that the genetic environment of NOD.H2^{h4} mice is not a predisposing factor to allow for increased iodine incorporation in Tg, under conditions of elevated iodine intake that induce ISAT.

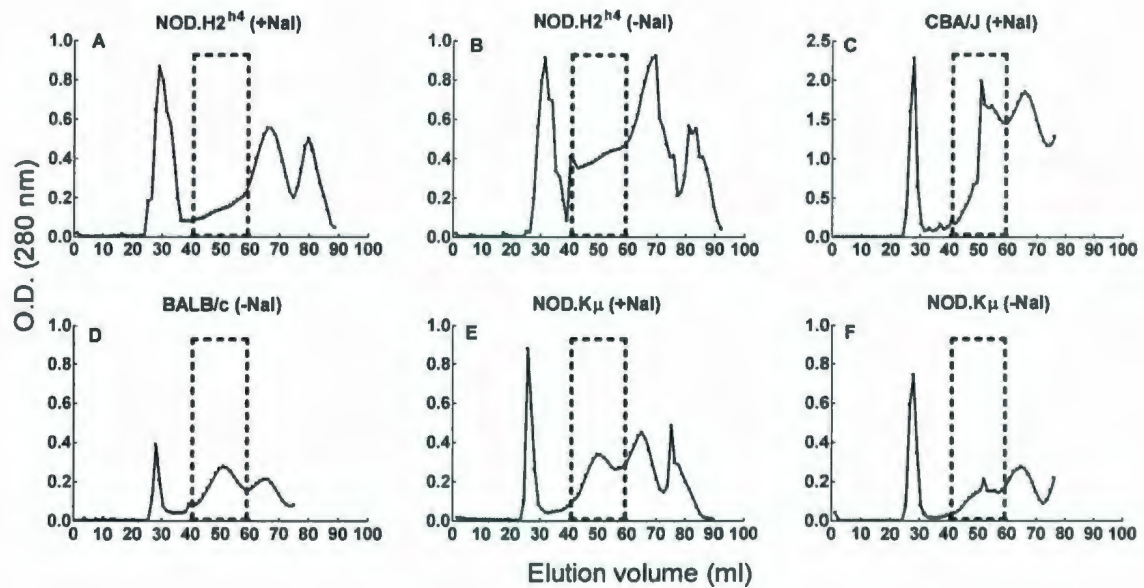


Fig. 1. Elution profiles of thyroid extracts subjected to gel filtration on a Sepharose CL-4B column. Thyroids (n=13, A, B, D, E and F; n=19, C), removed from 6-8 week-old mice placed on NaI-supplemented drinking water (+NaI) or normal water (-NaI), were homogenized in PBS buffer, pH=7, as described in Materials and Methods. Boxes indicate the elution volume of free Tg used to calibrate the column.

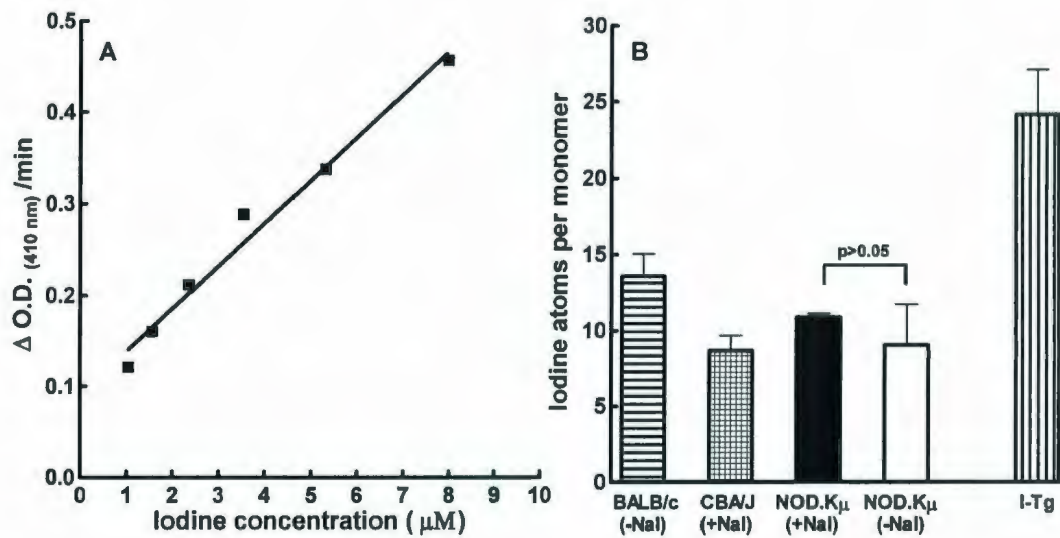


Fig. 2. Determination of iodine content in Tg samples. A) Representative standard curve showing the reduction of optical density at $\lambda=410$ nm of the iodine-catalyzed ceric-arsenite reaction as a function of T4 added. B) Iodine content of Tg preparations isolated as shown in Fig. 1, from thyroids of mice receiving NaI/drinking water (+NaI) or controls (-NaI). Each bar represents the mean iodine content of Tg \pm SD from two to three independent measurements. The last bar shows the iodine content of in vitro-iodinated Tg (I-Tg) purified from BALB/c mice and used as a target Ag in ELISA and cell proliferation assays. Statistical significance was calculated by the t test

3.3.2. Serum IgG responses to Tg or I-Tg preparations in ISAT

To seek immunological evidence that I-Tg formation occurs during ISAT development, we examined whether mice developing ISAT will generate IgG antibodies preferentially responding to I-Tg vs Tg. To this end, we first monitored the kinetics of ISAT development in our colony and observed a gradual increase in disease incidence with low levels of thyroid mononuclear cell infiltration 7-15 days following NaI supplementation (**Table 1**). Significant differences in both incidence and severity of ISAT were observed on d28, d42 and d77 after the initiation of NaI intake as shown (**Fig. 3**), with concomitant Tg-specific IgG responses that were significantly different between the experimental and control groups (**Table 1**). At a time when ISAT was well established, (day 42), the serum IgG responses from iodine-fed mice did not show preferential reactivity against I-Tg vs Tg (**Fig. 4A**). To exclude the possibility that the equivalent responses to these target molecules were not due to opposing effects of iodination on the antigenicity of Tg, i.e. blockade of prior B-cell epitopes and simultaneous formation of neoantigens by iodine atoms on Tg, we performed competition assays by ELISA. It was found that free Tg or I-Tg inhibited equivalently the IgG response to solid phase-bound Tg (**Fig. 4B**) or I-Tg (**Fig. 4C**). These data did not provide support for the notion that I-Tg forms a preferential autoimmune target in the course of ISAT development in NOD.H2^{h4} mice.

Table 1. ISAT development in NOD.H2^{h4} mice

Time	Diet ^a	Thyroiditis incidence	Mean Infiltration Index	Tg-specific IgG response ^c (O.D.405 nm at 1:50 serum dilution)
Day 7	NaI	2/8	0.38	0.11 ± 0.00
	Control	0/8	0.00	0.11 ± 0.01
Day 11	NaI	4/8	1.00	0.10 ± 0.00
	Control	1/8	0.50	0.13 ± 0.01
Day 15	NaI	5/8	1.38	0.25 ± 0.00
	Control	1/8	0.63	0.22 ± 0.01
Day 28 ^b	NaI	9/10	2.00	0.46 ± 0.05
	Control	0/10	0.00	0.10 ± 0.00
Day 42 ^b	NaI	9/10	2.20	0.96 ± 0.05
	Control	1/10	0.20	0.09 ± 0.00
Day 77 ^b	NaI	10/10	3.5	1.42 ± 0.11
	Control	3/10	0.8	0.11 ± 0.01

^a Seven to eight-week old NOD.H2^{h4} mice were placed on water supplemented with 0.05% NaI for the time periods described above. Three to six female and four to six male mice were sacrificed in each time period.

^b Significantly higher severity of ISAT in the iodine-fed group as assessed by the nonparametric Mann-Whitney U test (day 42, p=0.0007; day 77, p=0.0011).

^c ELISA results are expressed as mean OD values of triplicate wells ± SD. IgG responses to ovalbumin were at background levels.

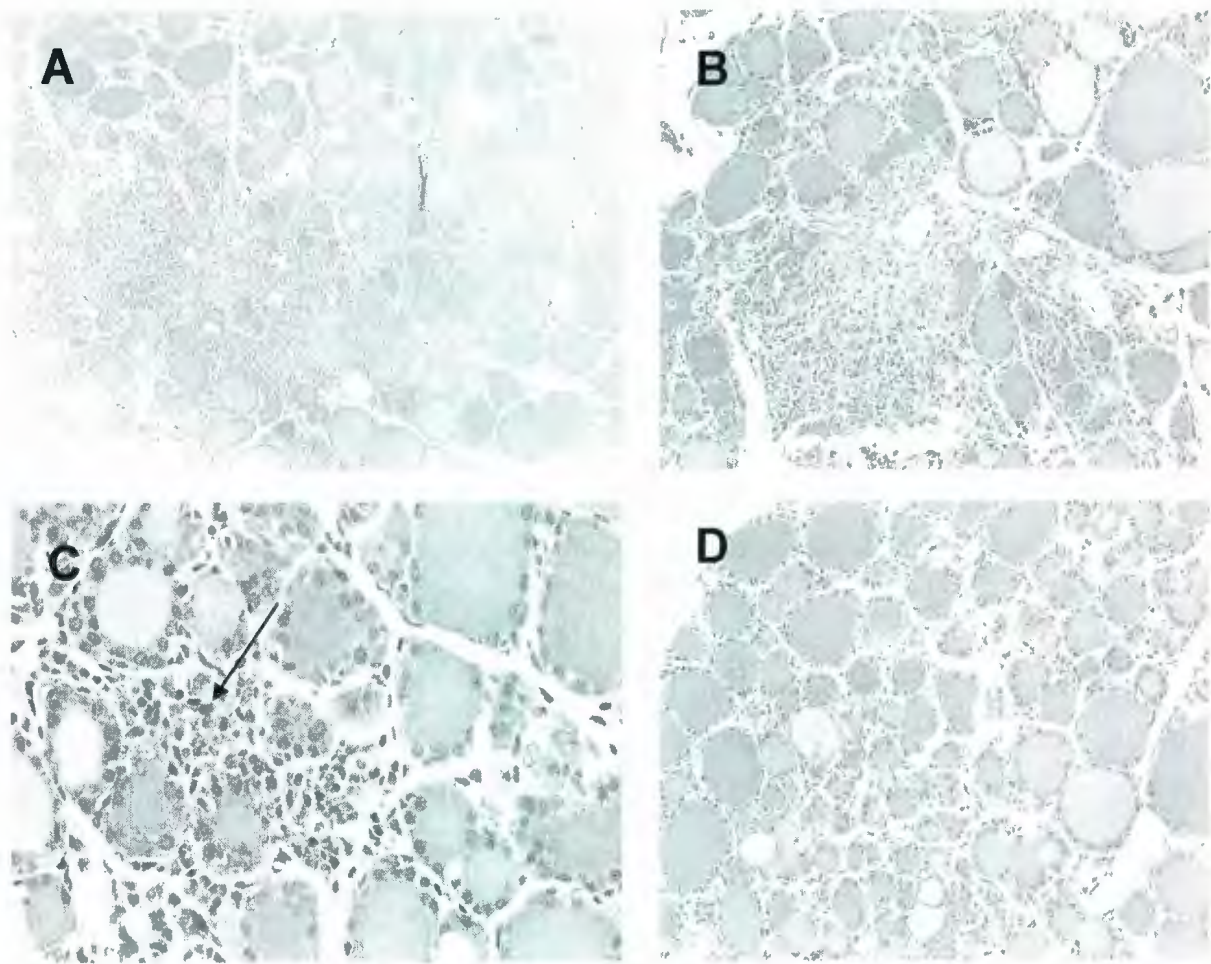


Fig. 3. Representative histological appearance of thyroid glands from iodine-fed (A-C) and control (D) NOD.H2^{h4} mice. Degrees of mononuclear cell infiltration: A) day 77 (infiltration index = 5), B) day 42 (infiltration index = 3), C) day 7 (infiltration index = 1) and D) day 7 (infiltration index = 0). Magnifications (A), 100 x; (B, D), 200 x; (C), 400 x.

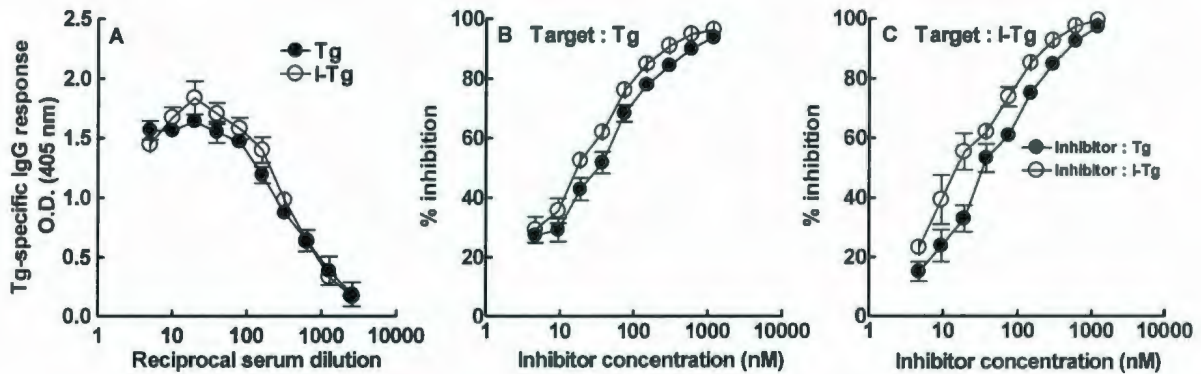


Fig. 4. A) Serum IgG responses to Tg (●) or I-Tg (○) preparations, assayed by ELISA. Pooled sera from 6 female and 5 male mice (day 42) were obtained and used at 1:5 starting dilution in PBS/Tween 20/0.1% BSA. Results are expressed as mean O.D. values of triplicate wells \pm S.D; B) Competitive inhibition of IgG binding to solid phase-bound Tg using Tg (●) or I-Tg (○) as an inhibitor; C) Competitive inhibition of IgG binding to solid phase-bound I-Tg using Tg (●) or I-Tg (○) as an inhibitor. Initial concentration of inhibitor was 1210 nM and sera were used at 1:200 dilution. O.D. values from wells without inhibitor ranged from 1.1-1.2. The inhibition using OVA as a control competitor did not exceed 8%.

3.3.3. Proliferative responses of NOD.H2^{h4} lymphoid cells to Tg and I-Tg during ISAT development

We next examined possible differences between Tg and I-Tg antigenicity at the T cell level, in early and late stages of ISAT development. NOD.H2^{h4} thyroid-draining cervical lymph node cells, isolated after 7 days of iodide supplementation, were cultured in the presence of Tg or I-Tg and their proliferative responses were examined. The S.I. values against either antigen were generally undetectable (S.I. <2), providing no evidence for differential reactivity to I-Tg in early stages of ISAT (**Fig. 5A**). After 28 or 42 days of iodide supplementation, the spleen cell responses to Tg and I-Tg were low but comparable (S.I. values between 2-4) (**Fig. 5B,C**). Collectively, the above data did not provide evidence that ISAT is associated with T-cell responses to putative I-Tg molecules in NOD.H2^{h4} mice.

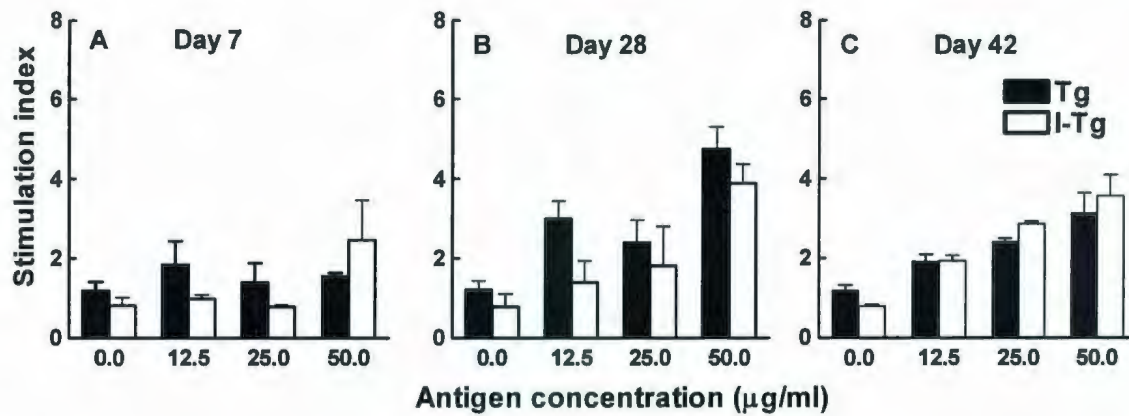


Fig. 5. Proliferative responses to Tg and I-Tg using thyroid-draining cervical lymph node cells (A) or splenic cells (B & C) (two mice per group), on the indicated days following initiation of iodide supplementation. Background levels were: A) 500 cpm, B) 1800 cpm and C) 760 cpm.

3.4. Discussion

The iodine-mediated acceleration of spontaneous thyroiditis in NOD.2H^{h4} mice has been well confirmed in several studies (Braley-Mullen et al., 1999; Hutchings et al., 1999; Rasooly et al., 1996) but the reasons behind this intriguing observation remain obscure. Since Tg serves as a storage molecule of available iodine, we have examined the iodine content of Tg in mice developing ISAT, seeking evidence for I-Tg formation in vivo that might be linked to the increased onset and severity of disease. Our study was initially hindered by the fact that the 660 kDa peak of soluble, free Tg was not discernible in chromatograms of thyroid extracts from NOD.2H^{h4} mice, especially those exhibiting both mild ISAT (mean I.I. = 2.00) and circulating Tg-specific serum IgG, four weeks after the initiation of the iodine-rich diet. The formation of larger than usual pellets in centrifuged thyroid extract samples prior to gel filtration suggested possible generation of Tg-Ab complexes either in vivo or during the Tg purification procedure. To circumvent this problem, we purified Tg from thyroids of B-cell deficient NOD.2H^{h4} mice (NOD.K μ ^{null}) which do not develop ISAT when they receive NaI in their drinking water (Braley-Mullen et al., 2000). We found no significant difference in the iodine content of Tg between control and experimental groups (9.0 ± 2.7 vs 10.9 ± 0.3 I atoms per monomeric Tg subunit). Our data are in apparent contrast with those of Sundick et al. who observed that CS chickens, which are susceptible to iodide-induced thyroiditis, incorporate at least 60 I atoms per Tg molecule when placed on a high iodide diet (20 mg KI/dl drinking water) (Sundick et al., 1987). The reasons behind this discrepancy are unclear, and the extensive immune dysregulation contributed by non-MHC genes in the NOD background (Anderson et al., 2005) precludes meaningful comparisons between the two models.

The fact that Tg-specific Abs are readily detected in ISAT (Braley-Mullen et al., 1999; Hutchings et al., 1999; Rasooly et al., 1996) prompted us to look for putative differences in B-cell reactivity against Tg vs I-Tg preparations. We hypothesized that chronic iodide consumption might lead to intrathyroidal generation of Tg with slightly higher than normal iodine content that might become a preferred target in the autoimmune response. Thus, I-Tg was prepared *in vitro* with approx. 24 I atoms per monomer and its antigenic profile was compared vs that of normal Tg in ELISA. After 7 weeks of iodide supplementation, i.e. at a time when ISAT is well developed, the serum IgG responses were equally strong against I-Tg vs Tg. Competitive inhibition assays provided no evidence that iodide in I-Tg blocked existing determinants or facilitated formation of neoantigenic epitopes in a manner that could skew the autoantibody response. These data did not provide indirect support for I-Tg generation in ISAT, although it cannot be formally excluded that Tg iodination *in vitro* may differ both qualitatively and quantitatively from the enzyme-mediated intrathyroidal iodination process.

The ease of detection of Tg-specific Abs in ISAT is in apparent contrast with the very low level of T-cell reactivity to Tg observed either in thyroid draining lymph nodes or spleens of NOD.H2^{h4} mice (Braley-Mullen et al., 1999; Verma et al., 2000). Our results confirm this finding, which is reminiscent of analogous observations regarding the insulin-specific responses in NOD mice developing spontaneous diabetes (Anderson et al., 2005; Kaufman et al., 2001). It has been reported that the natural autoantibody repertoire of NOD mice is highly active against self-antigens such as insulin, Tg and DNA (Thomas et al., 2002) and it is possible that genetic abnormalities in the regulation

of Tg-reactive B cells in NOD.H2^{h4} mice may contribute to autoimmunity. Iodide has also been reported to enhance IgG synthesis by human peripheral blood lymphocytes *in vitro* (Weetman et al., 1983). In our study, the detection of low but equivalent proliferative cell responses to Tg and I-Tg in lymphoid organs of NOD.H2^{h4} mice developing ISAT additionally suggested that the iodine content of Tg does not affect its antigenicity in this model.

Our inability to demonstrate formation of I-Tg in this ISAT model is seemingly at odds with the results of numerous EAT studies (Bagchi et al., 1985; Carayanniotis, 2007; Champion et al., 1987; Ebner et al., 1992; Ruwhof et al., 2001) attributing a critical role for Tg iodination in the disease process. In several studies, iodine-depleted Tg does not activate thyroiditogenic T cells but by raising the Tg iodine content to normal levels *in vitro* the immunopathogenicity of the molecule is re-established (Champion et al., 1987; Ebner et al., 1992). At the other end of the spectrum, highly iodinated Tg may cause EAT with increased incidence and severity in SJL mice (Dai et al., 2002), but when these mice are fed a high iodine diet, their Tg preparations do not show high iodine incorporation *ex vivo* and the mice develop goitrous hypothyroidism with no apparent autoimmune basis (Li et al., 2007). To our knowledge, *in vivo* formation of I-Tg in normal (i.e. non goitrous) mice exposed to a chronic iodine-rich diet has not yet been reported.

If enhanced iodine intake does not accelerate SAT via an I-Tg “link” in NOD.H2^{h4} mice what might be its mode of action? In this regard, it is worth mentioning that in the ISAT model a mouse consuming on average 5 ml of 0.05% NaI/water daily, receives

approximately 2 mg of iodide per day, i.e. a 2000-fold higher amount than that present in normal diet (Rose et al., 2002). This dietary regimen has been recently shown to cause damage in the ultrastructure of NOD.H2^{h4} thyroid epithelial cells associated with apoptosis and necrosis (Teng et al., 2009), a process that might be influenced by production of high H₂O₂ levels in NOD.H2^{h4} thyrocytes (Burek et al., 2008). In addition, enhanced iodine intake may upregulate adhesion molecules such as ICAM-1 on thyrocytes (Bonita et al., 2003) thus accelerating mononuclear cell infiltration into the thyroid. This process has been postulated to operate via the elevated generation of reactive oxygen intermediates (Burek et al., 2008; Sharma et al., 2008). However, the same dietary regimen does not appear to cause overt histological changes in the thyrocytes of other mouse strains - except for SJL (Li et al., 2007) - and the genetic factors predisposing NOD.H2^{h4} mice to ISAT remain unknown. Finally, iodine may amplify inherent defects in immune cells involved in the autoimmune response such as dendritic cells which have been reported to exhibit phenotypic and functional abnormalities in NOD.H2^{h4} mice as compared to dendritic cells from CBA mice (Strid et al., 2001). Iodine may also directly impair the function of regulatory T cells that have been known to contribute to ISAT resistance in NOD.H2^{h4} mice (Nagayama et al., 2007; Nakahara et al., 2009; Yu et al., 2006). Future research efforts in this area may yield new insights in the pathogenesis of thyroiditis.

Chapter 4: Tg-binding IgG antibodies found in NOD.H2^{h4} mice with ISAT do not belong to the natural autoantibody repertoire.

4.1. Abstract

High iodine intake has been reported to increase the incidence and severity of ISAT in NOD.H2^{h4} mice via an unknown mechanism. While Tg-binding IgG antibodies are readily detected in the sera of iodine-fed mice, T cell responses to Tg are very low or undetectable. To explain this phenomenon, we hypothesized that Tg-binding IgG belong to the natural autoantibody (NA) repertoire whose production may be T cell-independent. We examined our premise by testing the polyreactivity of Tg-binding IgG Ab against Tg, iodinated Tg (I-Tg), two self antigens (actin and DNA) and trinitrophenyl-ovalbumin (TNP-OVA) at three levels: the serum level, after serum fractionation on a protein-G column; and following purification of Tg-binding IgG antibodies. IgG and IgM responses were recorded against all antigens at the serum level and after serum fractionation in the experimental (NaI/H₂O) and control group. Our data showed that iodine administration increased mainly the IgG and not IgM responses to Tg and I-Tg whereas responses to actin, DNA and TNP-OVA were not influenced by the dietary regimen. Also, IgG and IgM Ab reacted similarly to Tg and I-Tg showing that I-Tg is not a preferential target over Tg. At the level of purified Tg-binding IgG, strong responses to Tg and I-Tg were recorded in the NaI/H₂O group while responses to other antigens were at background level (TNP-OVA) or undetectable (actin and DNA), showing lack of polyreactivity. In contrast, Tg-binding IgG from the control group were polyreactive responding to all antigens except DNA. These data indicate that Tg-binding IgG Ab detected in iodine-fed mice are likely not to belong to the NA subset and are produced by conventional B2 cells.

4.2. Introduction

NA are found in the sera of healthy individuals in the absence of antigenic stimulation (Avrameas et al., 2007). These antibodies are characterized by their ability to bind to many conserved self-antigens (Avrameas, 1991), with low affinity, they are polyreactive (Avrameas et al., 2007; Notkins, 2004) and they are encoded by non-mutated germline genes (Avrameas et al., 2007; Lacroix-Desmazes et al., 1998; Zelenay et al., 2007). Many issues regarding NA biology and function still remain unclear. It is not certain whether NA production is T cell-independent (Coutinho et al., 1995). Also, the relationship between NA and pathologic autoantibodies found in autoimmune diseases remains unknown (Avrameas, 1991). So far, studies have shown that some pathologic autoantibodies found in animal models of SLE and diabetes are polyreactive and they are identical, in terms of V gene usage, with NA supporting the view that pathologic autoantibodies are indistinguishable from NA (Avrameas, 1991; Quintana et al., 2004; Thomas et al., 2002).

NOD.H2^{h4} mice produce Tg-binding IgG autoantibodies by the age of 7 months (Braley-Mullen et al., 1999) and their presence is correlated with disease development (Braley-Mullen et al., 1999; Rasooly et al., 1996). High iodine intake induces the production of Tg-binding IgG serum antibodies via an unknown mechanism (Braley-Mullen et al., 1999; Rasooly et al., 1996). Despite the abundant production of Tg-binding IgG antibodies after NaI administration, T cell responses to Tg using splenocytes or cervical draining lymph node cells are very low or undetectable (Braley-Mullen et al., 1999; Verma et al., 2000). To explain this discrepancy, we hypothesized that Tg-binding IgG

serum antibodies in mice with ISAT belong in the NA group, generated by B1 cells that do not require T cell help for antibody production. To test our hypothesis, we examined whether Tg-binding IgG detected in NOD.H2^{h4} mice are polyreactive since one feature characterizing NA is the ability of antibodies to respond to many self-antigens. Screening was performed against Tg, iodinated Tg (I-Tg), two other self antigens (actin and DNA) and trinitrophenyl-ovalbumin (TNP-OVA). Antibody polyreactivity was assessed at three levels: a) the serum level; b) after serum fractionation on a protein-G column; and c) following purification of Tg-binding IgG antibodies by affinity chromatography on Tg-coupled Sepharose 4B.

4.3. Results

4.3.1. Serum IgG and IgM responses against a panel of antigens.

To examine serum IgG and IgM responses against Tg, I-Tg, actin, DNA and TNP-OVA, sera from thirteen 6-8 week old NOD.H2^{h4} mice were obtained after 28, 42 and 77 days of iodine supplementation and tested in an ELISA assay. In iodine-fed mice, very high but equivalent IgG responses to Tg (O.D. values 1.94-2.30) and I-Tg (O.D. values 2.12-2.15) were obtained on all days tested (**Table 2**) indicating that the dietary regimen did not render I-Tg a preferential target over Tg with a normal iodine content. Significantly lower yet considerable responses to the same antigens (O.D. values 0.46-1.68) were obtained in the control group confirming prior observations that iodine administration increases the production of Tg-reactive IgG in the serum. In the iodine-fed group, IgG responses to the self antigens actin and DNA were variable but did not increase over time indicating that high iodine intake does not generally associate with enhanced production of IgG reactive to self antigens. Similarly, the IgG response to TNP-OVA was high but unaffected by iodine intake. At the IgM level, comparable responses were again recorded against Tg and I-Tg in iodine-fed mice, and they tended to increase at the later time points (d42, d77) over the responses mounted by the control group (**Table 3**). Strong serum IgM responses to actin and DNA, as well as TNP-OVA were detected in both experimental and control groups and appeared unaffected by iodine consumption. In conclusion, high iodine intake in NOD.H2^{h4} mice “spontaneously” increased serum IgG – and to a much lesser extent IgM - specific for Tg or I-Tg, in a selective manner since this enhancing effect was not observed in the serum IgG and IgM responses to actin, DNA, or TNP-OVA.

Table 2. Serum IgG responses against a panel of antigens.

Time	Diet ^a	IgG response (O.D.405 nm at 1:10 serum dilution) ^b				
		Target Antigen				
		Tg	I-Tg	Actin	DNA	TNP-OVA
Day 28	NaI	1.94 ± 0.08	2.15 ± 0.05	0.17 ± 0.00	0.72 ± 0.03	0.57 ± 0.03
	Control	1.49 ± 0.00	1.46 ± 0.11	0.28 ± 0.00	0.15 ± 0.01	0.51 ± 0.04
Day 42	NaI	2.22 ± 0.16	2.11 ± 0.12	0.31 ± 0.01	0.61 ± 0.07	0.93 ± 0.06
	Control	1.68 ± 0.03	1.68 ± 0.08	0.41 ± 0.02	0.42 ± 0.03	0.85 ± 0.03
Day 77	NaI	2.30 ± 0.33	2.12 ± 0.36	0.29 ± 0.01	0.33 ± 0.00	1.02 ± 0.02
	Control	0.46 ± 0.03	0.53 ± 0.02	0.35 ± 0.01	0.81 ± 0.02	0.71 ± 0.02

^a Six to eight-week old NOD.H2^{h4} mice were placed on water supplemented with 0.05% NaI for the time periods shown. Due to animal losses, sera were obtained from six female and seven male mice on day 28 and 42 and five female and three male mice on day 77.

^b ELISA results are expressed as mean OD values of duplicate wells ± SD after 30 minute incubation with the substrate.

Table 3. Serum IgM responses against a panel of antigens.

Time	Diet ^a	IgM response (O.D.405 nm at 1:10 serum dilution) ^b				
		Target antigen				
		Tg	I-Tg	Actin	DNA	TNP-OVA
Day 28	NaI	0.14 ± 0.00	0.21 ± 0.01	0.68 ± 0.03	0.24 ± 0.00	0.75 ± 0.01
	Control	0.15 ± 0.02	0.14 ± 0.03	0.72 ± 0.10	0.32 ± 0.01	0.99 ± 0.02
Day 42	NaI	0.45 ± 0.00	0.67 ± 0.02	1.01 ± 0.07	0.43 ± 0.01	1.35 ± 0.08
	Control	0.26 ± 0.01	0.27 ± 0.00	0.87 ± 0.08	0.45 ± 0.03	1.29 ± 0.02
Day 77	NaI	0.46 ± 0.02	0.51 ± 0.02	0.92 ± 0.04	0.38 ± 0.04	1.23 ± 0.00
	Control	0.30 ± 0.02	0.34 ± 0.01	1.05 ± 0.02	0.70 ± 0.05	1.17 ± 0.08

^a Six to eight-week old NOD.H2^{h4} mice were placed on water supplemented with 0.05% NaI for the time periods shown. Due to animal losses, sera were obtained from six female and seven male mice on day 28 and 42 and five female and three male mice on day 77.

^b ELISA results are expressed as mean OD values of duplicate wells ± SD after 30 minute incubation with the substrate.

4.3.2. Testing the polyreactivity of IgG and IgM responses after fractionation on protein - G-Sepharose.

To examine IgG responses in the absence of potential inhibitory effects by serum IgM directed against the same target antigens, we purified IgG from pooled sera of NOD.H2^{h4} mice on a high iodine diet (d42 n=13 and d77 n=8) or controls (d42 n=13 and d77 n=8) and retested them by ELISA for reactivity to the same panel of antigens, as above. Removal of IgM did not alter the previous reactivity pattern since IgG from both control and experimental groups was easily detected against Tg or I-Tg and this response was significantly elevated by consumption of iodine (**Table 4**). In contrast, the same dietary regimen seemed to rather decrease the strong response to actin, DNA or TNP-OVA observed in IgG purified from control sera. This might indicate that polyreactive Tg-binding IgG are included in this group. IgG purified from pooled sera of CBA/J mice (n=15, additional control) gave background responses to Tg or I-Tg highlighting the propensity of the NOD.H2^{h4} background to respond to this thyroid antigen. The IgG-depleted eluate (containing IgM) was concentrated to the initial serum concentration and retested against the same antigen panel. High iodine intake did not increase the low IgM responses to Tg or I-Tg and seemed again to reduce rather than increase the considerable IgM responses to actin, DNA or TNP-OVA (**Table 5**). CBA/J-derived IgM responses against Tg or I-Tg were once more barely detectable, whereas the reactivity to the other antigens (in particular TNP-OVA) was strong. In conclusion, serum fractionation did not appear to alter the IgG or IgM reactivity profile observed in 4.3.1.

Table 4. Purified IgG responses against a panel of antigens.

Strain	Diet ^a	IgG response (O.D.405 nm at 10 µg/ml) ^b				
		Target Antigen				
		Tg	I-Tg	Actin	DNA	TNP-OVA
CBA/J	Plain water	0.05 ± 0.01	0.03 ± 0.00	0.29 ± 0.03	0.09 ± 0.00	0.83 ± 0.02
NOD.H2 ^{h4}	NaI water	1.33 ± 0.08	1.47 ± 0.04	0.62 ± 0.04	0.28 ± 0.01	1.26 ± 0.08
NOD.H2 ^{h4}	Plain water	0.59 ± 0.02	0.51 ± 0.00	1.11 ± 0.01	0.60 ± 0.01	1.84 ± 0.01

^a Six to eight-week old NOD.H2^{h4} mice were placed on water supplemented with or without 0.05% NaI.

^b Sera from 15 female 7-month old CBA/J mice were pooled and passed through a Protein G-Sepharose 4 column. Sera from NOD.H2^{h4} mice on water with or without NaI for 42 days (n=13 per group) and 77 (n=8 per group) days were pooled and IgG was purified. ELISA was performed using 100 µl of 10 µg/ml purified IgG from all groups. This IgG concentration was extrapolated defined from full titration curves of purified IgG (data not shown). ELISA results are expressed as mean OD values of duplicate wells ± SD after 30 minute incubation with the substrate.

Table 5. IgM responses against a panel of antigens after IgG depletion.

Strain	Diet ^a	IgM response (O.D.405 nm at 1:10 serum dilution) ^b				
		Target Antigen				
		Tg	I-Tg	Actin	DNA	TNP-OVA
CBA/J	Plain water	0.13 ± 0.02	0.09 ± 0.00	0.39 ± 0.02	0.37 ± 0.03	1.27 ± 0.08
NOD.H2 ^{h4}	NaI water	0.21 ± 0.03	0.20 ± 0.00	0.46 ± 0.04	0.32 ± 0.01	0.99 ± 0.02
NOD.H2 ^{h4}	Plain water	0.2 ± 0.00	0.19 ± 0.01	0.80 ± 0.01	0.51 ± 0.02	1.43 ± 0.01

^a Six to eight-week old NOD.H2^{h4} mice were placed on water supplemented with or without 0.05% NaI.

^b Sera from 15 female 7-month old CBA/J mice were pooled and passed through a Protein G-Sepharose 4 column. Sera from NOD.H2^{h4} mice on water with or without NaI for 42 (n=13 per group) and 77 (n=8 per group) days were pooled and IgG was purified. IgG-depleted fractions were concentrated to the initial serum volume. ELISA was performed using 1:10 dilution of IgG-depleted fractions from all groups. ELISA results are expressed as mean OD values of duplicate wells ± SD after 30 minute incubation with the substrate.

4.3.3. Testing the polyreactivity of Tg-binding IgG antibodies in ISAT.

To investigate whether Tg-binding IgG detected in iodine-fed NOD.H2^{h4} mice are polyreactive, purified IgG from NaI/H₂O and control group was passed through a Tg-coupled Sepharose 4B column and Tg-binding IgG was eluted and tested by ELISA against the same panel of antigens. In the experimental group, Tg-binding IgG reacted strongly to Tg and I-Tg while responses to other antigens were at background level (TNP-OVA) or undetectable (actin and DNA) (**Fig. 6A**) showing that increased iodine intake selectively enhances the response to Tg and I-Tg. These data suggest that Tg-binding IgG found in iodine-fed mice represent Tg-specific rather than NA IgG clones. In contrast, Tg-binding IgG from control mice were polyreactive since they responded significantly to TNP-OVA and weakly to Tg, I-Tg, and actin (**Fig. 6B**). The response to DNA was undetectable. Tg-binding IgG antibodies from the non-autoimmune-prone CBA/J strain were also used at a starting concentration of 10 µg/ml but responses to all antigens were at baseline (data not shown). Overall, the data revealed that the enhanced IgG-response to Tg in NOD.H2^{h4} mice following an iodine-rich diet does not draw significantly from the NA repertoire.

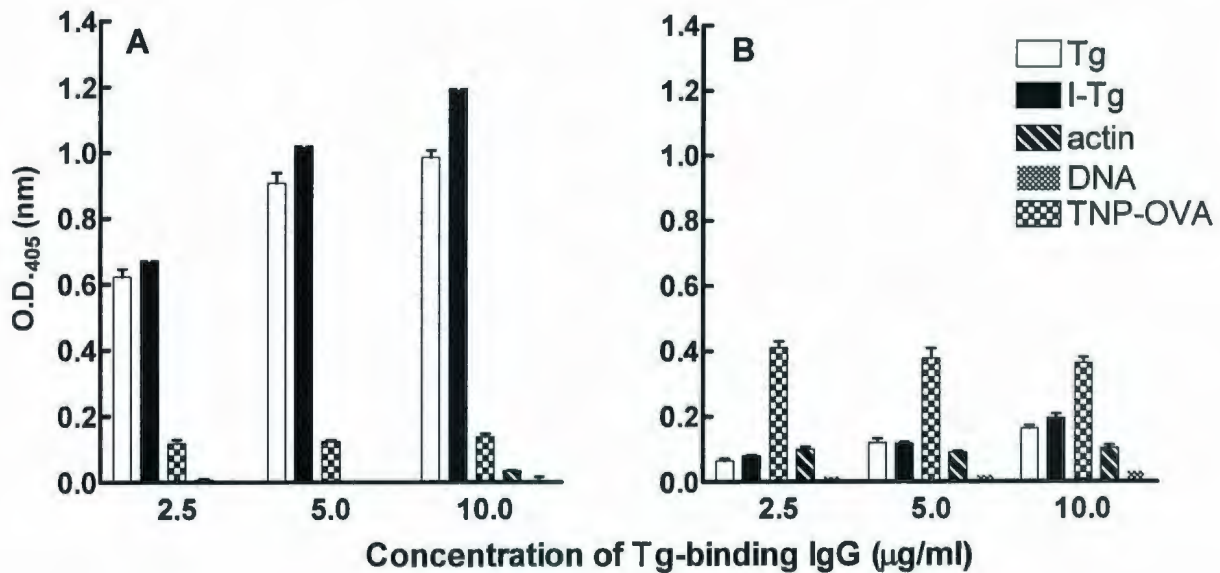


Fig. 6. Response of Tg-binding IgG antibodies, purified from sera of (A) iodine-fed or (B) control NOD.H2^{h4} mice. Tg-binding IgG was purified from pooled sera (day 42, n=11 and day 77, n=8 after the initiation of NaI intake) and used at a starting concentration of 10 µg/ml in an ELISA assay against the panel of antigens shown. Responses of 10 µg/ml of CBA/J-derived Tg-binding IgG to the panel of antigens were at baseline (data not shown). ELISA results are expressed as mean OD values of duplicate wells ± SD after 30 minute incubation with the substrate.

4.4. Discussion

High iodine intake has been reported to increase the incidence and severity of SAT in NOD.H2^{h4} mice as well as the production of Tg-binding IgG antibodies (Braley-Mullen et al., 1999; Rasooly et al., 1996). To explain the presence of high serum anti-Tg IgG titers in the apparent absence of detectable T cell responses to Tg (Braley-Mullen et al., 1999; Verma et al., 2000) in these mice, we hypothesized that these IgG might be derived from the NA repertoire. Since polyreactivity against a large number of self-antigens is a main characteristic of NA (Avrameas et al., 2007), we examined in this study the reactivity profile of Tg-binding IgG at three levels: at the serum level, at the purified IgG level-i.e. in the absence of IgM which can inhibit IgG responses to antigens (Adib et al., 1990) - and following purification of Tg-binding IgG. IgG and IgM responses were detected against all antigens at the serum level and after serum fractionation. A common feature observed at all levels was that IgG and IgM responses to Tg and I-Tg were not significantly different indicating that *in vitro* iodination of Tg did not generate epitopes preferentially recognized by Tg-binding IgG or IgM, confirming our data described in Chapter 3.

Previous studies have highlighted that the NA repertoire of the parental NOD mice has unique features (Thomas et al., 2002). Specifically, it was shown that the frequency of B cell hybridoma generation using splenocytes from naïve NOD mice was 5 - to 20-fold higher than in naïve BALB/c or B6 mice indicating that B cells in NOD mice are highly active. Further studies showed that NOD-derived anti-insulin IgG mAb recognized insulin with low avidity, reacted with other self antigens like DNA, Tg and cardiolipin

while their CDR3 regions were characterized by limited N segment diversity (Thomas et al., 2002). Our ELISA data, following purification of Tg-binding IgG from NOD.H2^{h4} control group, confirmed the presence of NA IgG clones in the sera of NOD.H2^{h4} mice (**Fig. 6B**) as previously shown in B/W mice (Adib et al., 1990) and non-autoimmune prone BALB/c mice (Adib et al., 1990; Berneman et al., 1992). The fact that Tg-binding IgG responded higher to TNP-OVA than Tg or I-Tg is in agreement with previous studies showing NA antibodies reacting higher with antigens other than those used for their purification (Adib et al., 1990; Berneman et al., 1992; Hentati et al., 1991). Also, responses to all antigens except DNA were characterized by low O.D. values as described previously (Berneman et al., 1992). Interestingly, high iodine intake did not increase equally responses to actin, DNA and TNP-OVA but selectively enhanced the IgG reactivity to Tg and I-Tg (**Fig. 6A**). This observation does not support the hypothesis that Tg-binding IgG from iodine-fed mice are derived from NA IgG clones since they are not polyreactive. Nevertheless, in the NaI/H₂O group, a very low amount of Tg-binding IgG may be recruited from NA because there is a low reactivity to TNP-OVA (**Fig. 6A**). This was confirmed in an additional assay, in which a low reactivity to actin was also observed (data not shown). Our data indicate that iodine does not generally trigger B cell clones to produce IgG against self and non self antigens but selectively stimulates B cells to produce mainly Tg-binding IgG and not IgM as previously reported (Braley-Mullen et al., 1999; Rasooly et al., 1996). This observation is in contrast to a previous *in vitro* study describing that iodine can increase non specifically antibody synthesis in human peripheral blood lymphocyte cultures stimulated with pokeweed mitogen compared to lymphocytes cultured with potassium chloride (Weetman et al., 1983). A possible

explanation for the selective stimulation of B cells in iodine-fed mice is that iodine triggers the initiation of an immune response against the thyroid via a still undefined mechanism and a consequence of this response is activation of B2 cells and production of Tg-specific IgG. Thus, Tg-specific IgG might be the result and not the cause of the immune response in ISAT. The production of Tg-specific IgG by B2 cells however, requires T cell help and the fact that spontaneous T cell responses to Tg are very low or undetectable cannot yet be understood.

The mode of action of iodine in ISAT development remains unknown. Previous studies have highlighted that thyrocytes from iodine-fed OS chickens (Bagchi et al., 1995), BB/W rats (Li et al., 1994), goitrous-NOD mice (Many et al., 1995) and NOD.H2^{h4} mice (Teng et al., 2009) acquire necrotic and apoptotic characteristics under electron microscopy. These observations suggest that excess iodine which is taken up by thyrocytes can induce the cell death of thyroid cells leading to release of autoantigen like Tg. Intrathyroidal DC might take up Tg and migrate to the thyroid draining cervical lymph nodes to initiate an immune response against the thyroid. In addition, iodine may accelerate ISAT by affecting the function of immune cells like DC which have been reported to have phenotypic and functional defects compared to DC from CBA/J mice (Strid et al., 2001). Furthermore, iodine may downregulate the function of Tregs which have a crucial role in ISAT development (Nagayama et al., 2007; Yu et al., 2006) either by acting directly on them or by changing the cytokine profile of iodine-treated APC that interact with Treg. Further studies will contribute to unmask the role of iodine in thyroiditis development in NOD.H2^{h4} mice.

4.5. Thesis summary

High iodine intake accelerates SAT development and production of Tg-specific IgG in NOD.H2^{h4} mice via an unknown mechanism. In this study, we examined whether excess iodine results in formation of Tg with high iodine content *in vivo*. In Chapter 3, our data suggested that the NOD.H2^{h4} genetic background does not promote increased iodine incorporation in Tg, following high iodine intake. Furthermore, *in vitro* generated I-Tg was equivalently targeted by IgG as Tg, indicating that iodine does not affect recognition of Tg epitopes by Tg-specific IgG in NOD.H2^{h4} mice. At the T cell level, similar responses were recorded against I-Tg and Tg supporting indirectly that I-Tg is not generated after excess iodine in NOD.H2^{h4} background.

In Chapter 4, it was examined whether Tg-binding IgG detected in iodine-fed NOD.H2^{h4} mice belong in the NA repertoire. Polyreactivity of Tg-binding IgG against Tg, I-Tg, actin, DNA and TNP-OVA was tested at three levels: at the serum level, at the purified IgG level and following purification of Tg-binding IgG. Responses to Tg and I-Tg were similar at all levels confirming that I-Tg is not selectively targeted by IgG or IgM. Iodine administration did not equally increase responses to actin, DNA and TNP-OVA as to Tg and I-Tg at all levels indicating that Tg-binding IgG do not belong in the NA repertoire and are produced by conventional B2 cells.

4.6. Future directions

Our data suggested that high iodine intake does not accelerate SAT development via the generation of Tg with high iodine content. To examine the mechanisms involved in ISAT development, we can investigate the possible effects of iodine treatment at different levels of the immune response and in particular, on the function of thyrocytes and immune cells like DC and Treg.

Hypothesis #1: Excess iodine can enhance the maturation and the antigen-presenting ability of DC allowing priming of autoreactive T cells.

According to Strid et al., bone marrow-derived DC from NOD.H2^{h4} mice generated in vitro fail to upregulate expression of MHC class II and co-stimulatory molecules after LPS treatment. Also, the same study has shown the inability of NOD.H2^{h4}-derived DC to activate a T cell hybridoma indicating that NOD.H2^{h4} - derived DC might have genetic defects (Strid et al., 2001). To examine our hypothesis, NOD.H2^{h4} bone marrow (BM)-derived DC will be cultured in the presence of GM-CSF (Li et al., 2006) and 9 days later iodine- in a range of concentrations that will not be toxic for the cells- will be added for an additional 24 hour of culture. Expression of MHC class II, CD80, CD86 and CD40 will be compared between iodine-treated DC and DC treated with GM-CSF alone by flow cytometry. If iodine-treated DC express higher levels of MHC class II and co-stimulatory molecules compared to untreated DC that would show that iodine can enhance DC maturation in vitro. Also, purification of DC from draining thyroid cervical

LNC of iodine-fed and control NOD.H2^{h4} mice will be performed and the expression of MHC class II and co-stimulatory molecules will be tested to examine whether iodine can promote DC maturation *in vivo*. DC from CBA/J mice will be used as control to examine whether possible iodine effects on DC *in vitro* or *in vivo* are NOD.H2^{h4}-specific. If we detect that DC from iodine-fed NOD.H2^{h4} mice have upregulated the expression of MHC class II and co-stimulatory molecules that would indicate that iodine can affect the maturation stage of DC *in vivo* supporting our hypothesis.

To investigate whether iodine treatment can enhance the antigen presenting ability of DC, *in vitro* iodine-treated and untreated BM-derived DC will be pulsed with a self antigen, Tg or a foreign antigen, OVA in the presence of purified CD4⁺ T cells from LNC of Tg or OVA-primed NOD.H2^{h4} mice. If significantly higher T cell proliferation is seen in the iodine-treated DC culture compared to the untreated DC culture that would indicate that iodine treatment can enhance the antigen presenting ability of DC *in vitro*. The same system using purified DC from iodine-fed and control mice can be used to examine whether iodine enhances the antigen presenting function of DC *in vivo*. Positive results will support our hypothesis indicating that iodine can enhance the antigen presenting ability of DC *in vitro* or *in vivo*.

Hypothesis #2: Excess iodine can downregulate the suppressive function of Tg-specific Treg allowing proliferation of effector T cells.

To examine our hypothesis, NOD.H2^{h4} Tg-specific Tregs will be generated as previously described (Verginis et al., 2005). Briefly, TNF- α -treated, semi-mature Tg-pulsed DC will be injected in NOD.H2^{h4} mice and 21 days later, CD4⁺CD25⁺ splenocytes will be purified based on magnetic cell sorting. Phenotypic analysis-expression of CD62L, FoxP3 and glucocorticoid-induced TNF receptor- as well as functional characteristics i.e. ability to suppress proliferation of effector T cells will be examined to confirm that the expanded cells are Tregs. Afterwards, Tregs will be cultured for 24 hours in the presence of iodine. Subsequently, their ability to suppress the proliferation of CD4⁺CD25⁻ effector T cells isolated from LNC of Tg-primed NOD.H2^{h4} mice, in the presence of mitomycin C-treated syngeneic splenocytes (APC) and Tg will be examined in a mixing experiment. We expect to observe that iodine-treated Tregs will not suppress or suppress less the proliferation of effector T cells compared to iodine-untreated Tregs indicating that iodine can partially or completely inhibit Treg function acting directly on them in vitro. To examine whether iodine can downregulate Treg function indirectly through cytokines produced by iodine-treated DC, in vitro iodine treated BM-derived DC or DC purified from cervical lymph nodes of iodine-fed NOD.H2^{h4} mice will be used as APC in a mixing experiment as previously described. If Tregs cultured with iodine-treated DC or DC from iodine-fed mice cannot suppress or suppress less than Tregs cultured with

untreated DC that would indicate that iodine treatment in vitro or in vivo can lead to the production of cytokines by DC able to inhibit Treg function.

Hypothesis 3: Excess iodine can induce cell death in NOD.H2^{h4} thyrocytes leading to release of Tg and high mobility group protein 1.

Previous studies have reported that human thyrocytes cultured in vitro in the presence of excess iodine are characterized by a necrotic or apoptotic phenotype (Golstein et al., 1996; Many et al., 1992). Also, thyrocytes from OS chickens administered 250 µg iodine intraperitoneally (Bagchi et al., 1995), iodine-fed BB/W rats for 12 weeks (Li et al., 1994) and iodine-fed goitrous-NOD mice for 4 days (Many et al., 1995) had developed necrotic characteristics after electron microscopy observation. Thyrocytes from NOD.H2^{h4} mice on NaI-H₂O were also described to have undergone apoptotic and necrotic changes after 8 to 24 weeks of iodine treatment (Teng et al., 2009). To examine our hypothesis, thyrocytes from 4-week old NOD.H2^{h4} mice will be isolated as previously described (Li et al., 2006) and will be cultured for 6 days in the presence of complete F-12 medium. On the seventh day, iodine concentration ranging from 10⁻⁵-10⁻³ M will be added to the culture for 24 hours. Flow cytometry will be used to estimate the percentage of cell death in our culture after labeling thyrocytes with Annexin-V to stain apoptotic cells and propidium iodide to stain necrotic cells. Electron microscopy will be used to confirm the presence of necrotic and apoptotic cells. Thyrocytes from the non-autoimmune prone CBA/J and BALB/c mice will be treated with or without iodine and will be used as control. If our hypothesis is correct, we expect to detect cell death only in

iodine-treated NOD.H2^{h4} thyrocytes. These data will indicate that thyrocytes from the NOD.H2^{h4} strain are susceptible to undergo apoptosis or necrosis in the presence of excess iodine.

To investigate whether Tg and high mobility group protein 1 (HMGB1) - a signal released from dying cells able to participate in DC activation and maturation (Green et al., 2009) - can be released by the iodine-treated NOD.H2^{h4} thyrocytes, thyrocyte culture supernatant after iodine treatment will be collected. The presence of Tg in the supernatant will be detected using a sandwich ELISA assay while the presence of HMGB1 will be detected in the supernatant of iodine-treated thyrocytes using Western blot. Supernatant will also be collected from the culture of iodine-untreated NOD.H2^{h4} thyrocytes as well as thyrocytes from CBA/J and BALB/c mice on a NaI diet or not. If our hypothesis is correct, we expect to detect Tg and HMGB1 in the supernatant of the culture of iodine-treated NOD.H2^{h4} thyrocytes indicating that an autoantigen, Tg and a danger-associated molecular pattern, HMGB1 can be released from the NOD.H2^{h4} thyrocyte after iodine treatment in vitro.

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