PARAMETERS INFLUENCING GENERATION OF CRYPTIC PATHOGENIC T-CELL EPITOPES IN THYROGLOBULIN

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BY

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ABSTRACT

Thyroglobulin (Tg) is a major autoantigen in autoimmune thyroiditis, a T-cell-mediated, organ-specific autoimmune disorder. Immunization with Tg or some Tg peptides can activate thyroiditogenic T cells and cause experimental autoimmune thyroiditis (EAT) in mice. Most of the identified pathogenic Tg peptides have been characterized as cryptic epitopes due to their insufficient presentation following processing of Tg in vivo and in vitro. The main goal of this study was to examine whether modification of Tg processing could enhance generation of cryptic T-cell epitopes. Two physiologically relevant parameters that could modify Tg processing were tested: the first was the formation of immune complexes (IC) of Tg with Tg-specific Abs; and the other was the increased iodination of Tg.

It is shown that generation of the non-dominant pathogenic Tg epitope (2549-2560), containing thyroxine (T4) at position 2553 (T4(2553)), is augmented in APC that internalize ICs of Tg with selected Tg-specific IgG mAbs. The enhancing effects of certain mAbs on the generation of T4(2553) is FcR-dependent, but FcR-mediated internalization of Tg is not sufficient for generation of T4(2553). The data rather suggest a mAb effect on Tg processing within APC. Interestingly, one of Tg-specific mAbs, 55H8, suppressed the generation of T4(2553) following processing Tg-mAb ICs. The suppressive role of 55H8 on activation of T4(2553)-specific T-cells was further investigated. It was found that 55H8 can bind to the

T4(2553) peptide within A^k MHC and prevent recognition of the peptide by T4(2553)-specific T-cells. These two studies indicate that Tg-specific Abs may regulate the course of AT by enhancing or suppressing activation of Tg-reactive pathogenic T-cells. It was shown that highly iodinated thyroglobulin (I-Tg) exhibits higher immunopathogenicity than normal Tg in mice. Processing of I-Tg in LNC in vivo, or in macrophages and dendritic cells, but not B cells, in vitro, promoted presentation of the cryptic pathogenic Tg peptide (2495-2511) to T cells. This study demonstrates that generation of non-iodinated, cryptic but pathogenic determinants during processing of I-Tg, may contribute to the development of AT. In conclusion, the studies describe general mechanisms as to how autoantibodies may regulate process of T-cell-mediated autoimmune diseases and how environmental factors such as excess iodine may trigger autoimmune responses.

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ABBREVIATIONS

a.a.	amino acid
Ab	antibody
Ag	antigen
AchR	acetylcholine receptor
ADCC	antibody-dependent cell-mediated cytotoxicity
AITD	autoimmune thyroid diseases
APC	antigen presenting cells
APL	altered peptide ligand
AT	autoimmune thyroiditis
ATCC	American Type Culture Collection
BB/W	Bio breeding/Worchester rat
BcR	B-cell receptor
CFA	complete Freund's adjuvant
CLIP	class II-associated invariant chain peptide
Con A	concanavalin A
CS	Cornell strain chicken
CTL	cytotoxic T lymphocyte
CTLL	cytotoxic T lymphocyte line
DC	dendritic cells
DIT	diiodotyrosine
DP	double positive thymocytes
EAE	experimental autoimmune encephalomyelitis
EAT	experimental autoimmune thyroiditis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FcγR	Fc-gamma receptor

GD	Graves' disease
HEL	hen-egg lysozyme
HI-Tg	high iodine thyroglobulin
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HT	Hashimoto's thyroiditis
IC	immune complex
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
I-Tg	iodinated thyroglobulin
LI-Tg	low iodine thyroglobulin
LNC	lymph node cells
LPS	lipopolysaccharide
LT	lymphocytic thyroiditis
MIIC	MHC class II compartments
mAb	monoclonal antibody
MBP	myeline basic protein
MHC	major histocompatability complexes
mIg	membrane immunoglobulin
MIT	monoiodotyrosine
NK	natural killer cell
NOD	non-obese diabetes
OS	obese strain chicken
OVA	ovalbumin
PCD	programmed cell death
PLP	proteolipid protein
RSA	rabbit serum albumin
SAT	spontaneous autoimmune thyroiditis

s.c.	subcutaneous
S.I.	stimulation index
T0(2553)	Tg peptide 2549-2560 with thyronine at position 2553
T3	triiodothyronine
T4	thyroxine
T4(2553)	Tg peptide 2549-2560 with thyroxine at position 2553
TcR	T-cell receptor
Th	helper T lymphocyte
THAA	thyroid hormone autoantibodies
Tg	thyroglobulin
TGB	thyroxine-binding-globulin
TPO	thyroid peroxidase
TSH	thyroid stimulating hormone
TT	tetanus toxin

CHAPTER 1

INTRODUCTION

1.1. Autoimmune thyroiditis

1.1.1. Autoimmune Hashimoto's thyroiditis

The human autoimmune thyroid diseases (AITD) include Hashimoto's thyroiditis (HT) and Graves' disease (GD). HT, also named autoimmune thyroiditis (AT) or lymphocytic thyroiditis (LT), is generally present in either a goitrous form (classic HT) or a non-goitrous form (atrophic thyroiditis) (Davies, 1994). Thyroid destruction occurs progressively over months to years during the course of HT. Although the levels of the thyroidal hormones, triiodothyronine (T3) and thyroxine (T4), may be within the normal range for some HT patients, the serum thyroid stimulating hormone (TSH) level can be elevated, indicating a sub-clinical thyroid failure (Vlase and Davies, 1999). GD can be divided into two types. One is the classical hyperthyroid GD, which is characterized by a decreased level of serum TSH and some degree of thyrotoxicosis – a clinical syndrome of hyperthyroidism resulting from increased serum T3 and T4 concentrations. Another is the euthyroidal GD with a normal TSH level and no thyrotoxicosis (Davies, 1994). The following paragraphs will discuss the immunopathology and the immunogenetics of HT.

The immunopathology of HT is highlighted with lymphocytic infiltration of the thyroid gland and production of autoantibodies (auto-Abs) against thyroidal autoantigens (auto-Ags). Lymphocytic infiltration ranges from mild focal to extensive. Focal thyroidal lymphocytic infiltration is found very frequently at autopsy, occurring in 40% of women and 20% of men (Okayasu et al., 1993). Diffuse lymphocytic infiltration occurs commonly in HT patients and causes various degrees of goiter. The infiltrating cells include plasma cells, macrophages and T-cells (Bigazzi and Rose, 1985). Formation of germinal centers and fibrosis can be seen in some thyroid glands from HT patients. Both CD4 and CD8 positive T-cells are found among the infiltrating cells (Bigazzi and Rose, 1985).

Two major thyroidal auto-Ags are thyroglobulin (Tg) and thyroid peroxidase (TPO). Auto-Abs against Tg and TPO occur in most HT patients (Weetman and McGregor, 1984). Serum anti-Tg and anti-TPO Abs have long been recognized as important indicators for HT diagnosis, but the presence of anti-Tg and anti-TPO auto-Abs does not necessarily indicate occurrence of HT. A study estimated that about 5-10% of healthy people have auto-Abs against thyroidal Ags (Bigazzi, 1993). The prevalence of anti-Tg and anti-TPO auto-Abs in normal populations seems to vary with age. A study conducted in the UK showed that 10.6% of healthy women aged 18-24 were positive for anti-Tg auto-Abs, but the frequency increased to 30.3% among women aged 55-64. Similarly, the frequency of anti-TPO auto-Abs increased with age increases, and the respective frequency for the two groups of women was 14.9% versus 24.2% (Prentice et al., 1990). HT patients

without anti-Tg or anti-TPO auto-Abs are rare. Depending on the methods used for detection of the serum auto-Abs, the frequency of auto-Ab positive HT patients can vary from 60% to 100% (Bigazzi, 1993). Using a sensitive radioimmunoassay, circulating auto-Abs against Tg and TPO were detected in 86-100% of patients with HT (Bigazzi and Rose, 1985). It is not clear whether or how these auto-Abs mediate the pathogenesis of HT, but such a high frequency of auto-Abs in HT patients clearly demonstrates a strong association between the anti-Tg/TPO auto-Abs and development of HT. (Burek and Bresler, 1990).

HT has a multi-factorial etiology, involving both genetic and environmental factors (Burek and Bresler, 1990). Clustering of HT in families is frequently observed, and concordance of HT between twins is significantly higher than between non-twin siblings, suggesting the importance of genetic predisposition in development of HT (Weetman and McGregor, 1984). Most of the genetic association and linkage analyses focus on human leukocyte antigen (HLA) genes, and have initially shown some degree of association between HT and specific HLA alleles (Weetman and McGregor, 1984). In some studies, HLA-DR3 is shown to associate with HT (Stenszky et al., 1987; Tandon et al., 1991). Other studies have shown a high risk of development of HT among the HLA-DR5 gene carriers (Farid, 1987; Weissel et al., 1980). HLA-DR4 gene has also shown a strong association with goitrous HT in Canadian people with a relative risk index of 5 (Thompson and Farid, 1985). However, one study has demonstrated that HLA class II polymorphism contributed little to the development of HT (Jenkins et al.,

1992). Since the results of the genetic association of HT with HLA alleles are not consistent in different studies, it is possible that the HLA genes may not be a major contributor to HT susceptibility (Roman et al., 1992). The variable results in different studies may reflect different ethnicities, case ascertainment, study methodologies and/or environmental influences (Weetman and McGregor, 1994).

Given the weak association of HLA genes with HT, other genes may regulate the susceptibility to HT, such as the germline genes coding the T-cell receptor (TcR) α- and β- chains. Several studies have examined the association between TcR Vβ-genes and HT development. One study showed a significant association of HT with TcR β-chain genes (Ito et al., 1989), but this association could not be confirmed in another study (Mangklabruks et al., 1991). The influence of immunoglobulin (Ig) genes on the development of HT is unclear, as few studies conducted showed conflicting results (Weetman and McGregor, 1994). Women are at 5-10 fold higher risk than men to develop HT, with a peak incidence between 50-60 years age (Vanderpump et al., 1995), suggesting that sex hormones may play a critical role in HT. Iodine is considered the most important environmental factor in HT induction (Rose et al., 1997). Either iodine deficiency or iodine excess may cause thyroid disorders (Woeber, 1991).

1.1.2. Animal models for AT

1.1.2.1. Induced experimental autoimmune thyroiditis

In 1956, Witebsky and Rose performed a pioneering study. They successfully induced experimental autoimmune thyroiditis (EAT), which showed similar immunopathology as HT, in rabbits by immunization with rabbit thyroid gland homogenate emulsified in complete Freund's adjuvant (CFA) (Witebsky and Rose, 1956). Following this protocol, EAT has been established in various animal species such as dog and guinea pig (Terplan et al., 1960), rat (Jones and Roitt, 1961), monkey (Kite et al., 1966) and mouse (Rose et al., 1971). Both homologous and heterologous Tg preparations have been used as immunogens to induce EAT in mice (Romball and Weigle, 1984). Generally, heterologous Tg preparations are more immunogenic but less thyroiditogenic than self Tg as detected by proliferation of draining lymph node cells (LNC) and thyroid histology. This suggests that certain unique epitopes within the homologous Tg molecule may be more thyroiditogenic than those shared between self Tg and the heterologous Tgs (Kong and Lewis, 1990).

EAT has also been induced in some strains of mice by immunization with Tg peptides emulsified in CFA (Carayanniotis and Rao, 1997), or TPO protein and its peptides (Kotani et al., 1990; Kotani et al., 1992). A strong adjuvant such as lipopolysaccharide (LPS) or CFA is required for inducing an immune response against the thyroidal Ags and their peptides. However, Balasa et al. successfully induced mouse EAT using Tg conjugated to a monoclonal antibody (mAb)

specific for mouse class II MHC molecules as Ag, without addition of a adjuvant (Balasa and Carayanniotis, 1993). Other approaches can also induce EAT in animal models. Braley-Mullen et al. established an EAT mouse model by adoptively transferring Tg-primed and activated splenocytes into normal syngeneic recipients (Braley-Mullen et al., 1985). Purified Tg-specific T-cells or T-cell clones have been reported to induce EAT in syngeneic mouse recipients (Okayasu, 1985; Romball and Weigle, 1987), supporting the view that EAT is T-cell mediated autoimmune disease. Thymectomy plus total body sublethal irradiation of young mice can trigger autoimmune response against their thyroid glands (Penhale et al., 1973), but it is not known whether the autoimmune response is restricted only to the thyroid glands.

Quite commonly, EAT is induced in mice immunized with purified mouse Tg emulsified in CFA. Through the use of this model, some questions related to immunopathology and immunoregulation of autoimmune thyroiditis have been extensively addressed (Kong, 1999). To examine the association of major histocompatability complex molecules (MHC) with EAT induction, Rose's group immunized 33 different mouse strains of 11 different MHC haplotypes with thyroid extracts emulsified in CFA. The susceptibility of the mouse strains was classified based on the incidence and severity of thyroidal lymphocytic infiltration. Mice carrying H-$2^{s,\ k,\ and\ q}$ MHC haplotypes are the most susceptible to EAT induction, strains with H-$2^{a,\ m,\ r,\ and\ p}$ MHC haplotypes have intermediate susceptibility, and mice with H-$2^{b,\ d,\ g,\ i,\ and\ v}$ MHC haplotypes are

resistant to EAT induction (Vladutiu and Rose, 1971b). To examine the association of HLA genes with EAT induction in animal models, Kong et al. introduced the HT-associated HLA gene DR3 into the EAT-resistant mouse strain B10.M, and found that over 40% of the DR3-transgenic B10.M mice developed severe EAT following immunization with Tg (Kong et al., 1996).

The roles of Th1/Th2 cells and their secreting cytokines such as IFN-γ and IL-4 on induction of EAT have also been addressed in the adoptive transfer EAT model described above (Stull et al., 1992). It is generally accepted that organ-specific T-cell-mediated autoimmune diseases such as EAT and experimental autoimmune encephalomyelitis (EAE) are induced by Ag-specific Th1 cells, and induction of a Th2 response may ameliorate the diseases (O'Garra et al., 1997; Pearson and McDevitt, 1999). Interestingly, Tg-primed T-cells from mice lacking the IFN-γ gene could induce EAT in recipient mice (Tang et al., 1998b), suggesting that Tg-specific effector T-cells can cause thyroid destruction without IFN-γ secretion. In addition, disruption of the IL-4 gene in Tg-primed effector T-cells did not exacerbate EAT in recipient mice (Tang et al., 1998a), indicating IL-4 is not essential for development of EAT. Culturing Tg-primed lymphocytes with IL-4 before adoptive transfer did not benefit the recipients in respect of thyroid destruction albeit a decrease of anti-Tg Ab production was observed in the recipients. In contrast, culturing Tg-primed lymphocytes with IL-10 significantly decreased their thyroiditogenicity and prevented development of EAT in recipient mice {Mignon-Godefroy and Charreire, 1995}. Furthermore, IL-12 can

promote generation of thyroiditogenic T-cells in vitro that, subsequently, adoptively transfer EAT to recipient mice {Braley-Mullen and Bickel}.

1.1.2.2. Animal models for spontaneous autoimmune thyroiditis

Spontaneous autoimmune thyroiditis (SAT) is characterized by spontaneous development of thyroidal lymphocytic infiltration, production of anti-thyroid auto-Abs and cell-mediated destruction of thyroidal tissue. These immuno-pathological changes closely resemble those in HT. Several animal models of SAT have been established. The most extensively studied model is the Obese strain (OS) chicken, which develops SAT during the first few weeks of life (Wick et al., 1971). The disease is characterized with severe mononuclear cell infiltration, formation of germinal centers in thyroid glands and development of auto-Abs against Tg, T3 and T4. The autoimmune thyroidal destruction leads to hypothyroidism and necessitates a hormone-replacement treatment of the younger chickens for successful breeding and propagation of the strain (Wick et al., 1989). Increase iodine uptake exacerbates the thyroid destruction in OS chickens (Bagchi et al., 1985).

The Bio Breeding/Worchester (BB/W) rat was originally used to study spontaneous induction of insulin-dependent diabetes mellitus (IDDM), but some of the rats have also been found to develop hypothyroidism and SAT at 60 to 120 days of age (Allen et al., 1986). The incidence of development of spontaneous

IDDM and SAT in the BB/W rat is very high. Up to 59% of rats with IDDM also have lymphocytic infiltration in thyroid glands (Sternthal et al., 1981). SAT has also been reported in the Buffalo rat. Over 26% of old Buffalo rats show high titers of anti-thyroid auto-Abs and mononuclear cell infiltration in thyroid glands, and neonatal thymectomy increases the incidence (Noble et al., 1976). SAT has also been found in non-obese diabetes (NOD) mice, a well-established model for spontaneous IDDM. The incidence of SAT in these diabetes-prone mice ranges from 67 to 90% (Bernard et al., 1992). It is possible that common mechanisms of organ-specific autoimmune destruction may exist between SAT and IDDM in the NOD mice.

1.1.3. Autoimmune responses against Tg

1.1.3.1. Mapping T-cell epitopes in Tg

The Tg molecule is a 660 KD homodimeric glycoprotein, which is stored in the thyroid follicular lumen and comprises over 75% of the dry weight of thyroid gland (Rayner et al., 1993). Cloning and sequencing of the cDNA of Tg from AKR/J mice indicated a primary sequence of 2746 amino acid (a.a.) residues, preceded by a 20 a.a. signal peptide (Kim et al., 1998). Tg is unique in its content of iodinated tyrosine residues. Normal human Tg varies widely in iodine content from 0.1% to 1.1% (Taurog, 1996). The main function of Tg is to act as the thyroid prohormone. The thyroid hormones, thyroxine and triiodothyronine, are synthesized following thyroid peroxidase-catalyzed modification or iodination of the tyrosine residues within Tg (Rayner et al., 1993).

Thyroid peroxidase (TPO) and Tg are the two major autoantigens in AT (Weetman 1994). TPO peptides 100-119, 420-434, 625-644, and 882-901 are the most common sites recognized by TPO T-cell line, indicating that they may be immunogenic epitopes in AT {Fisfalen 1995}. One T-cell epitope of TPO 118-131 has been identified as pathogenic in EAT and shares part of its sequence with Tg, providing an evidence of epitope spreading {Hoshioka 1993}. Our study focuses on examining the autoimmune responses against Tg by using the Tg-indued EAT mouse model. Since Tg has a large molecular size and extensive modifications

such as glycosylation and iodination, the task of mapping pathogenic epitopes in Tg is extremely challenging. Diverse strategies have been used for this purpose. Champion et al. found that poorly iodinated Tg failed to elicit EAT in mice and could not activate Tg-specific T-cell hybridomas (Champion et al., 1987). This observation raised the possibility that dominant pathogenic Tg epitopes might be iodinated. T-cell epitope mapping efforts led to the identification of a thyroiditogenic T4-containing Tg epitope, T4(2553), which contains a thyroxine molecule at position 2553 (Champion et al., 1991; Hutchings et al., 1992). The T4(2553)-reactive T-cell hybridomas were originally created by fusion of the Tg-primed LNC with thymoma cells (Rayner et al., 1987). This study emphasized the role of the hormonogenic sites in the pathogenicity of Tg.

Other approaches have been taken by several labs to map Tg epitopes. Using trypsinized Tg fragments, Texier et al. identified a 40mer Tg peptide (1672-1711) that could stimulate the cytolytic activity of Tg-primed CTL and induce EAT in CBA mice following immunization with the peptide (Texier et al., 1992). Based on the hypothesis that Tg may share similar epitopes with TPO, Hoshioka et al. identified a 14mer Tg peptide (2730-43) as an immunodominant epitope, which could stimulate proliferation of Tg-primed LNC. However, the 14mer peptide failed to induce EAT following immunization of animals with the peptide and adjuvant (Hoshioka et al., 1993). A different approach has been taken by Carayanniotis' group. The rat Tg sequence was screened for sites that were likely to contain T-cell epitopes based on the "AMPHI" and "tetramer motif" computer

algorithms (Rothbard and Taylor, 1988; Taylor, 1994). The predicted Tg sites were synthesized as 12-18 mer peptides, and then, used to challenge EAT-susceptible mice for induction of EAT. Following this strategy, two Tg peptides, 2495-2511 and 2694-2711, were identified as pathogenic determinants (Carayanniotis et al., 1994; Chronopoulou and Carayanniotis, 1992). However, both peptides were characterized as non-dominant T-cell epitopes, since in vitro, they could not stimulate Tg-primed LNC to proliferate, and in vivo, they could not prime T-cells able to recognize intact Tg in a subsequent proliferation assay in vitro (Carayanniotis and Rao, 1997).

1.1.3.2. Anti-Tg auto-Abs

Tg-specific Abs were first reported in HT patients in 1958 (Roitt and Doniach, 1958). The frequency of anti-Tg auto-Abs in healthy women varies depending on the method used for Ab measurement: from 5-10% by haemagglutination and ELISA to 15-20% by RIA, while the frequency in men is 10-fold lower than in women (Beever et al., 1989) (Vanderpump et al., 1995). Tg-specific Abs have been found in the majority of patients with HT, and their occurrence in HT patients is commonly associated with anti-TPO auto-Abs (Nordyke et al., 1993). It is currently not clear whether or how anti-Tg auto-Abs are involved in the pathogenesis of HT, or whether they are non-pathogenic Abs generated secondary to the T-cell infiltration and tissue damage in the thyroid gland. Tg-specific Abs cannot fix complement, but may play a role in antibody-dependent

cell-mediated cytotoxicity (ADCC) (Weetman et al., 1989). Both healthy people and AT patients may carry anti-Tg Abs in their circulation. It seems that some of the anti-Tg auto-Ab clones may be harmless and others may be pathogenic and related to the development of HT (Tomer, 1997).

Various approaches have been taken to identify the specificity of anti-Tg auto-Abs. Enzyme-digested fragments of Tg molecules have been used in epitope mapping (Male et al., 1985), but this approach can only identify linear, not conformational epitopes. In order to overcome this, cross-inhibition studies have been performed by using panels of anti-Tg mAbs (Piechaczyk et al., 1987; Ruf et al., 1983). These studies suggest that most disease-associated anti-Tg auto-Abs may be restricted in their epitopic specificity, in other words, they are oligoclonal, while the natural anti-Tg auto-Abs in healthy people may be polyclonal (Dietrich et al., 1991). In addition, Dong et al. reported that the binding sites of pathogenic anti-Tg Abs were clustered on certain regions within Tg, but the epitopes of the natural anti-Tg Abs were spread randomly within Tg (Dong et al., 1989). Recently, Prentice et al. has also demonstrated that the Tg epitopes recognized by Tg-specific Abs from AT patients are different from those recognized by Abs from healthy individuals (Prentice et al., 1995). Similarly, Tomer suggested that the oligoclonal anti-Tg auto-Abs of HT patients have different specificity from the polyclonal anti-Tg auto-Abs of healthy people, and might participate in the initiation of HT (Tomer, 1997).

To examine the direct thyroiditogenicity of Tg-specific Abs, several workers attempted to elicit EAT by passive transfer of sera. Successful induction of EAT by transferring sera from Tg-immunized animals to syngeneic recipients has been reported in different animal models (Nakamura and Weigle, 1969; Polley et al., 1981; Tomazic and Rose, 1975; Vladutiu and Rose, 1971a). Also, mice injected with the hybridoma cells secreting anti-Tg mAb developed thyroid lesions quickly (Yokochi et al., 1991). However, there are other conflicting studies that show that Abs or Tg-immuned serum cannot directly induce EAT (Inoue et al., 1993). The reason(s) for these discrepancies remains unkown. The mechanism underlying Ab-mediated pathogenesis is also unknown. It has been proposed that Abs that bind to Tg may enhance Tg internalization and epitope presentation on Ag presenting cells (APC), and thus, to activate auto-reactive T-cells specific to Tg peptides (McIntosh and Weetman, 1997). This hypothesis provides a mechanism to bridge Ab-mediated pathogenesis with T-cell-mediated thyroidal destruction.

1.2. The role of iodine in autoimmune thyroiditis

1.2.1. Iodine metabolism and thyroid hormone synthesis

Iodine is a trace element necessary for thyroid hormone synthesis. T4 and T3 are formed following an intramolecular coupling of two iodotyrosyl residues in Tg (Woeber, 1991). Hormone synthesis can be divided into the following steps: active transport of iodide; iodination of tyrosyl residues of Tg; coupling of iodotyrosine molecules within Tg to form T4 and T3; proteolysis of Tg to release iodotyrosine and iodothyronines, and secretion of iodothyronines; deiodination of iodotyrosines and rescue of iodide (Taurog, 1996). These processes are discussed in the next few paragraphs.

Iodine transport and iodination of Tg molecules

Iodine is actively transported into the thyroid follicle cells against both a chemical and electrical gradient (Wolff, 1964). The iodine transport across the plasma membrane of the follicle cells is dependent on cellular ATP, and the Na^+-K^+ ATPase may be involved in this process (Wolff, 1964). Binding of phospholipids to iodine can make the iodine soluble, and thus, a membrane carrier has been suggested to mediate the iodine transportation (Weiss et al., 1984). The most important regulator for iodine transportation is thyroid stimulating hormone (TSH), which enhances iodine uptake by the follicular cells

(Halmi et al., 1960). Iodination of Tg is a post-translational modification that occurs after the maturation of Tg molecules. It takes place at the cell-colloid interface close to the apical membrane of the thyroid follicular cells and requires the presence of four elements, TPO, H_2O_2, iodide and Tg (Taurog, 1996). The reaction of iodination is catalyzed by TPO, which is excreted together with Tg to the apical membrane by the thyroid follicular cells. TPO within the intracellular exocytotic vesicles is not functional because of the lack of accessibility to H_2O_2, which is a necessary component for TPO activity and is present only near the apical membrane of the follicular cells (Taurog, 1996). This site-restricted activity of TPO minimizes non-specific iodination of the intracellular protein molecules.

Formation of thyroid hormones

TPO contains two binding sites, one for iodine binding and the other for tyrosine binding (Woeber, 1991). The tyrosine oxidation reaction catalyzed by TPO yields some free radicals and iodotyrosines, monoiodotyrosine (MIT) and diiodotyrosine (DIT). In addition to catalyzing the iodination of tyrosine residues, TPO is also able to catalyze intracellular coupling of two iodotyrosyl residues to form T4 and T3 within the Tg molecule. Formation of DIT and MIT may facilitate a coupling event (Deme et al., 1975). Generally, several conserved sites within Tg are used for hormone synthesis such as tyrosine residues at position 5, 2553, 2567 etc (Dunn and Dunn, 1999). However, many other sites have also been described as tyrosyl donors or recipients for hormone synthesis (Marriq et al., 1989; Ohmiya et al., 1990). The primary sequence and the native

structure of Tg molecule may play a major role in selecting which tyrosine residues are used for hormonogenesis (Maurizis et al., 1981). The mature Tg molecules containing T4 or T3 are stored in the follicular lumen for future use.

Proteolysis of Tg molecules and release of hormones

To release thyroid hormone, the mature hormone-containing Tg molecules within the follicular lumen must be endocytosed by the surrounding follicular cells and further digested in the cells to free the T4 and T3 molecules. The follicular cells take up the stored Tg molecules either through fluid phase pinocytosis or a receptor-mediated specific uptake (Consiglio et al., 1981). Following endocytosis, the endocytotic vesicles fuse with lysosomes, where Tg molecules meet the proteases. The lysosomal enzymes play a major role in the intrathyroidal degradation of Tg. Some proteolytic enzymes such as cathepsins are crucial for generation of T4 and T3 hormones (Dunn et al., 1991). Once released from Tg, the T4 and T3 hormones rapidly leave the follicular cells and enter the circulation by crossing the basal membrane of the cells. TSH stimulates Tg endocytosis and hormone release (Dunn, 1996). Most of the released iodotyrosine residues following Tg degradation are deiodinated within the follicular cells, and the iodide is recycled for iodination of new Tg molecules.

1.2.2. The relationship between iodine excess and Hashimoto's thyroiditis

The minimum daily iodine requirement is about 50 μg (Woeber, 1991). The thyroid gland can defend a short-term wide fluctuation in iodine supply to retain a constant secretion of the thyroid hormones. However, chronic iodine excess or deficiency may lead to some thyroid dysfunction and possibly thyroid disease (Rose et al., 1997). Total daily urine iodine less than 25 μg may be considered as iodine deficiency (Woeber, 1991). The most common pathologic consequence of iodine deficiency is endemic goiter. A more severe deficiency may cause endemic cretinism in fetal life and childhood. Although environmental iodine deficiency continues to be a major public health problem in some developing countries, it is mostly eradicated in Europe and North America due to the iodization program that adds additional iodine into salt or oil (Brush, 1952; Hetzel and Dunn, 1989). The program significantly prevented endemic goiter in iodine-deficient areas and communities (Pharoah and Connolly, 1987), and was beneficial as a therapy for the patients with spontaneous hyperthyroidism (Azizi, 1985). However, a converse problem, iodine excess, arose due to the abuse of iodized products and iodine-containing drugs (Oddie et al., 1970; Woeber, 1991). In 1980, the recommended daily allowance (RDA) for iodine intake was 150 μg in the US, but many Americans are ingesting over 1 mg of iodine per day (Barsano, 1981). A survey in 1970 showed that the average iodine intake by Americans varied from 240 μg to 740 μg (Oddie et al., 1970).

At least two different types of iodine excess can cause dysfunction of the thyroid gland. One is a persistent long period of exposure to a moderately increased iodine level such as that resulting from the abuse of iodized salt or oil. Another is a short-term exposure to a large dose of iodine contained in certain drugs. Increased iodine uptake by iodine-deficient people resulted in a direct toxic effect on the thyroid function and subsequent thyroiditis (Mahmoud et al., 1986). The incidence of HT grew in some previously iodine-deficient areas after the Chernobyl explosion, perhaps due to the increased environmental iodine isotopes (Vermiglio et al., 1999). In 1971, Braverman and his colleagues examined the effect of excess iodine on euthyroid HT patients. Four out of seven patients developed severe hypothyroidism, suggesting a strong relation between excess iodine and HT (Braverman et al., 1971). A causative role of excess iodine on the development of HT was suggested based on both clinical and epidemiological observations (Kahaly et al., 1998; Konno et al., 1994; Vono et al., 1996). Some clinical diagnostic or therapeutic protocols may cause excessive iodine intake by patients. One example is amiodarone, an iodine-rich drug used frequently in the treatment of *Cardiac arrhythmias* and *Angina pectoris*. It is clear that long periods of taking amiodarone can induce anti-thyroid Abs and cause HT (Martino et al., 1984; Monteiro et al., 1986). However, a short-term exposure to a large dose of iodine may not be as dangerous as long-term exposure to a moderate dose of iodine, and it ordinarily does not lead to dysfunction of thyroid glands. Radioiodine-containing reagents used for clinical diagnosis and therapy may also cause HT, but the mechanism may be different from non-radioactive iodine

excess. It has been shown that radioactive iodine may cause a direct injury of thyroid tissue (Fisher and Oddie, 1969; Oddie et al., 1968).

1.2.3. Iodine-induced AT in animal models

In 1969, Evans et al. examined the effect of excess iodine on the development of AT in dogs. The dogs were immunized with dog thyroid extract, and some of them were simultaneously treated with excess iodine. The results demonstrated that dogs treated with excess iodine developed more severe thyroiditis than untreated dogs following immunization with the thyroid extract (Evans et al., 1969). Belshaw et al. confirmed the role of excess iodine on development of AT in iodine-deficient dogs treated with thiouracil. Most of the iodine-deficient animals developed severe lymphocytic infiltration in the thyroid tissue following treatment with excess iodine in the diet (Belshaw et al., 1974).

In 1985, Bagchi et al. demonstrated a role of excess iodine in induction of AT in chickens (Bagchi et al., 1985). Cornell strain (CS) chickens were chosen for the study, because the CS chicken is closely related to the OS chicken, which developes SAT, whereas the CS chicken does not. During the experiment, the CS chickens were treated from the first day of hatching with excess iodine through a daily supply of KI-containing water. Sera were taken from the iodine-treated chickens at different periods, and the titers of anti-Tg, T3 and T4 Abs were

determined. At 10 weeks of age, the chickens were sacrificed for histological examination of their thyroid glands. Those chickens that received excess iodine had up to 7.3 % lymphocytic infiltration as well as a high incidence of anti-Tg, T3 and T4 Abs. In contrast, only 1.28 % of chicken with normal iodine intake showed thyroid lymphocytic infiltration and a low incidence of Ab response. To further address the effect of iodine deficiency in development of AT, they raised the OS chickens under an iodine-deficient environment, and found that the incidence of presenting anti-Tg, T3 and T4 Abs was significantly decreased in these SAT-prone chickens. The data clearly demonstrated that excess diet iodine intake increased the incidence of AT in CS chickens.

Similar results have been found in the BB/W rat SAT model (Allen et al., 1986). The diabetic-prone BB/W rat develops SAT without functional hypothyroidism between 60 and 120 days of age. To examine the effect of excess iodine in the development of SAT in the BB/W rat, young rats less than 30 days of age were treated with water containing 0.05% of iodine for 7 - 8 wks. Serum anti-Tg Ab and the thyroid glands were examined to estimate the development of SAT. The data showed that rats treated with iodine-containing water had a significant higher incidence of SAT (77%) than those rats treated with tap water (30%). However, hypothyroidism did not occur in these iodine-treated rats as indicated by a normal T4, T3 and TSH levels, but occurred only when the young rats were hemithyroidectomized prior to iodine treatment (Allen et al., 1987), suggesting some other determinants may contribute to the development of hypothyroidism

in the iodide-treated BB/W rats. Neonatally thymectomized Buffalo rats have also been shown to be susceptibile to excess iodine-induced SAT and hypothyroidism (Allen and Braverman, 1990). After treating the thymectomized rats with iodine-containing water for 12 weeks, the rats exhibited a significantly higher incidence in development of SAT than the non-iodine-treated group (73% vs. 31%) as defined by thyroid lymphocytic infiltration. A mouse SAT model, NOD-H-2^{h4}, has also been used to examine the effect of excess iodine in the development of SAT (Rasooly et al., 1996). After feeding the mice with water containing 0.05% iodine for 8 wks, approximately 54% of the female mice and 70% of the male mice developed thyroid lesions, whereas only 1 out of 20 control mice that received plain water developed thyroiditis at the same age.

1.2.4. Iodination of Tg increases its immunogenicity

1.2.4.1. Circulating Tg has lower iodine content than intrathyroidal Tg

The thyroid gland is not an immunologically privileged site, and Tg molecules and its fragments circulate continuously in the blood. These circulating Tg molecules contain very little or are devoid of iodine, as compared to the stored Tg molecules in the thyroid follicles (Ikekubo et al., 1981; Schneider et al., 1983). It has been suggested that serum Tg is derived from the leakage of the colloid Tg through the lymphatic vessels (Daniel et al., 1967). This suggestion may not be

correct, since, otherwise, the circulating Tg would have contained similar iodine content as the stored Tg within the colloid. An observation by Chambard et al. may help to solve the puzzle of why the circulating Tg has much less iodine than the intrathyroidal Tg. They found that Tg synthesized within the follicular cells could be secreted through the basolateral membrane directly into the blood (Chambard et al., 1987). Since iodination only occurs at the apical membrane as described before, the newly synthesized Tg entering into the blood directly through the basolateral membrane would not contain any iodine and thyroid hormone. A transcellular vesicular transport (transcytosis) of Tg from the lumen to the basolateral membrane and into the blood occurring within the thyroid follicular cells may count for some of iodine content within the circulating Tg (Herzog, 1983). However, the transcytosis must occur at a very low rate since most of the endocytosed Tg molecules are degraded within the lysosomes to release T3 and T4 hormones.

Circulating Tg molecules from patients with some thyroidal abnormalities contain higher iodine content than that from normal individuals (Druetta et al., 1999). However, in patients with thyroid tumors, the iodine content of the circulating Tg is unchanged although an increased concentration of circulating Tg is observed (Schneider et al., 1983). The destruction of the thyroid follicles during the course of thyroid disease may result in leakage of the colloid Tg into the blood or lymphoid system. TSH stimulation of thyrocytes causes a marked increase of the iodine content in circulating Tg to a level similar to that of the

intrathyroidal Tg, suggesting that TSH stimulation may facilitate the release of iodinated Tg (Schneider et al., 1985). In addition, excess intake of diet iodine generally results in an increase of iodine content in Tg extracted from thyroid glands (Sundick et al., 1987).

1.2.4.2. The effects of iodine content on the immunogenicity of Tg

It has been suggested that iodination of Tg increases its immunogenicity and causes an autoimmune response against the iodine-modified Tg (Champion et al., 1992; Rose et al., 1997). Highly iodinated Tg molecules (HI-Tg) differ from low iodinated Tg (LI-Tg) in their conformational structure (Berg and Eckholm, 1975) and their susceptibility to thyroidal hydrolytic proteases (Fouchier et al., 1983; Lamas and Ingbar, 1978), indicating that iodination may alter the immunogenic properties of the Tg molecules.

Sundick et al. examined Ab responses against Tg preparations with different iodine content (Sundick et al., 1987). The HI-Tg preparation was obtained from thyroid glands of CS chicken fed with iodine rich diet and contained about 60 iodine atoms per Tg molecule. The LI-Tg preparation was purified from the thyroid glands of CS chickens treated with propylthiouracil, an inhibitor of iodination, and the iodine level of this LI-Tg preparation was undetectable. To compare their immunogenicity, they challenged CS chicken with either HI-Tg or LI-Tg. A stronger anti-Tg response was induced after immunization with HI-Tg

but not with LI-Tg, suggesting that iodination increases the immunogenicity of Tg. Similar results had been obtained in BB/W rats (Ebner et al., 1992). In this study, LI-Tg was obtained from rats fed with 0.5% methimazole, and the immunogenicity and thyroiditogenicity of this LI-Tg was compared with that of Tg purified from rats fed with normal diets. The BB/W rats rarely develop SAT before the age of 75 days. Young rats were immunized with LI-Tg or normal Tg emulsified in CFA and sacrificed before the age of 65 days for examination of anti-Tg Ab titers and thyroidal lymphocytic infiltration. About 31% of young BB/W rats developed EAT after immunization with normal Tg, whereas none of the rats developed EAT following immunization with LI-Tg. Also, the titer of anti-Tg Abs was higher in the sera from rats immunized with normal Tg vs. that in the sera from rats immunized with LI-Tg.

T-cells play a major role in the development of AT in animal models (Kong, 1999) and also possibly in human HT (Weetman. A. P., 1998). It has long been suspected that iodine modification may break the T-cell tolerance against Tg to initiate autoimmune thyroiditis. Allen et al. compared the relative frequency of HI-Tg-specific vs. LI-Tg-specific precursor T-cells in the AT-prone BB/W rats (Allen and Thupari, 1995). Circulating naïve T-cells from the young BB/W rats were tested for their activation by HI-Tg or LI-Tg in vitro. They found that there was no difference in the proliferation of these T-cells against HI-Tg and LI-Tg, suggesting that T-cell repertoire contains a similar frequency of precursor T-cells specific for HI-Tg and LI-Tg.

Using two Tg-specific T-cell hybridoma clones (Champion et al., 1985), Champion et al. demonstrated that activation of the T-cell hybridomas required the presence of iodine within Tg preparations (Champion et al., 1987). Tg purified from mice treated with aminotriazole was not able to stimulate the T-cell hybridomas to proliferate. In contrast, Tg from normal mice strongly activated the hybridoma cells. Immunization of mice with various Tg preparations confirmed that Tg devoid of iodine was not thyroiditogenic, but normal Tg induced significant EAT. Furthermore, human Tg with variable T4/T3 content activated the T-cell hybridomas to various degrees, and the activation indices correlated with the hormone content of the Tg preparations (Champion et al., 1987). The study suggested that iodinated determinants recognized by T-cells may play a central pathogenic role in the induction of EAT. The subsequent studies identified the specificity of the T-cell hybridomas to a T4-containing Tg peptide T4(2553) (Champion et al., 1991), and immunization with this peptide can directly induce EAT in susceptible mice (Hutchings et al., 1992). T4 was a crucial residue for recognition of the peptide by the T-cell hybridomas, since an analog peptide carrying a tyrosine instead of T4 at position 2553 did not activate the T-cells (Dawe et al., 1996). To examine whether iodine is important for the T4(2553) peptide to be pathogenic, Kong et al. compared the immunogenicity of the T4(2553) peptide with its non-iodine analog, T0(2553), which has the same peptide backbone as T4(2553) peptide, Tg2549-2560, but no iodine atoms on the phenyl-rings of the T4 molecule (Kong et al., 1995). They

demonstrated that both T4(2553) and T0(2553) peptides are immunogenic and suggested that the presence of iodine within T4 is not necessary for the thyroiditogenicity of the T4(2553) peptide.

In human studies, Rasooly et al. first described that iodine was required for appropriate recognition of Tg by human T-cells (Rasooly et al., 1998). A minimal amount of iodine was necessary for Tg to elicit proliferation of lymphocytes from HT patients. However, the study also showed that lymphocytes from healthy people or HT patients proliferated against iodinated Tg in a similar fashion. This was not expected since the frequency of Tg-reactive T-cells in HT patients should have been higher than that in normal individuals. In contrast to Rasooly's findings, Shimojo et al. found that the iodine content of Tg did not correlate with its activity to stimulate peripheral lymphocytes to proliferate, and also sera from AT patients reacted equally well to different Tg preparations regardless of their iodine content (Shimojo et al., 1988). The reasons for the discrepancies between the two studies remain obscure.

1.3. Cryptic T-cell epitopes and autoimmunity

1.3.1. Immunodominance and crypticity

When we immunize a host with a particular protein Ag, the host's T-cell response against the Ag usually focuses on a few sites within the protein molecule, and the response to other sites of the molecule is relatively insignificant. This phenomenon is denoted as immunodominance (Berzofsky, 1988). Immunodominance was first observed in the immune responses against foreign Ags such as hen-egg lysozyme (HEL), hemagglutinin (HA) and allergens. It was found that LNC from animals immunized with these foreign Ags only proliferated against a few synthetic peptides originating from the protein Ags (Finnegan et al., 1986; Hurwitz et al., 1984; Katz et al., 1982; Kurisaki et al., 1986). The concept of crypticity was introduced in 1993 by Sercarz, and it means the failure of certain potential determinants within a protein Ag to be recognized by T-cells following immunization with the protein Ag (Sercarz et al., 1993). T-cell epitopes of a protein Ag can be predicted by algorithm-based computation programs (Rothbard and Taylor, 1988; Sette et al., 1989b), but the number of predicted epitopes is generally much greater than that of the dominant epitopes mapped by T-cell proliferation and activation assays (Chronopoulou and Carayanniotis, 1992). Some of these predicted epitopes may have the capacity to induce significant immunological responses after challenging animals with the epitopes themselves, but be unable to be recognized by LNC previously primed

with the intact Ag, and thus, they are named as cryptic epitopes. A functional definition for immunodominance and crypticity is shown in **Table 1.1.**

Table 1.1. A functional definition for dominant and cryptic T-cell epitopes

In vivo immunization	In vitro LNC proliferation against			
	Intact Ag	Peptide#1	Peptide#2	Peptide#3
Intact Ag	+++	+++	+	-
Peptide#1	+++	+++	-	-
Peptide#2	+/-	-	+++	-
Peptide#3	-	-	-	+++

Table 1.1. Definition for immunodominant and cryptic T-cell epitopes. A dominant epitope, such as peptide #1, can strongly stimulate LNC primed with the intact Ag to proliferate. A cryptic epitope, like peptide #3, can induce a strong immune response, but it can neither stimulate the intact Ag-primed LNC to proliferate in vitro, nor induce T-cells recognizing the intact Ag in vitro. Peptide #2 is characterized as subdominant epitope for its intermediate capacity to activate intact-Ag-primed LNC.

Three parameters play a major role in selecting which peptide within a protein Ag acts as the immunodominant target. The first is the peptide affinity of binding to the specific MHC. The second is the availability of T-cells reactive to the peptide-MHC complexes (Sercarz et al., 1993). The third is the relative efficiency of a peptide to be generated and presented on professional APC such as DC and macrophages. Factors that affect the efficiency of Ag processing and

presentation will be discussed in following paragraphs, including primary and secondary structures of the Ag, MHC hyplotypes and differential Ag processing in diverse APC.

1.3.1.1. Primary and secondary structures of protein Ag affect peptide presentation

The amino acid (a.a.) sequence of a protein Ag is an essential factor that instructs Ag processing and generation of MHC-binding peptides. If a peptide sequence within the Ag encompasses a MHC-binding motif, the peptide can potentially be immunogenic. Amino acid substitutions within the peptide can increase or decrease its overall binding affinity to the MHC molecule (Sloan-Lancaster and Allen, 1996), (Jardetzky et al., 1990). Altered peptide ligands (APL) with a.a. substitutions at various positions can be used to predict the binding motif for a given MHC molecule, which then can be used for prediction of the potential MHC-binding sequences within a given protein Ag (Buus et al., 1987). However, binding to the MHC does not necessarily render a peptide immunogenic, because the peptide may not be generated during the digestion of the intact protein within APC or not recognized by T-cells (Sette et al., 1989b).

The a.a. sequence flanking a MHC-binding motif may also be important in affecting presentation of the peptide following processing of the protein Ag (Kurlander et al., 1999; Latek et al., 2000; Moudgil et al., 1996). The flanking

regions may contain critical sites for protein-unfolding and enzymatic digestion. Amino acid substitutions at these sites may prevent the enzymatic cutting to generate the MHC-binding fragments, or inhibit peptide loading into the MHC-binding groove.

The tertiary structure of a protein Ag may also influence the processing of the Ag in APC (Glimcher et al., 1983). For example, stabilization of HEL with additional intracellular disulphide-bridge-links can render HEL resistant to proteolysis and lead to a strong reduction in its immunogenicity (So et al., 1997); Also, dimerization of monomeric human chorionic gonadotropin (HCG) can alter the tertiary structure of the protein and thus affect presentation of T-cell epitopes during HCG processing (Rouas et al., 1993).

1.3.1.2. MHC-guided Ag processing and presentation

Exogenous Ags can be internalized by APC either through non-specific endocytosis such as pinocytosis and phagocytosis or by specific receptor-mediated endocytosis (Watts, 1997). Following internalization, the exogenous Ags enter the various stage endosomes where they meet local proteases and become partially digested. Digestion is completed in the MHC class II compartments (MIIC) which are formed after fusion of the late-stage endosomes with the enzyme-rich lysosomes. Within the MIIC compartments, the newly synthesized MHC class II molecules are chaperoned with HLA-DM molecules

and stabilized with the class II-associated invariant chain peptide (CLIP) derived from Ii chain, and a dynamic peptide exchange occurs for loading an appropriate peptide into the MHC-binding groove. Once a kinetic balance is achieved, the MHC-peptide complexes are stabilized and dissociate from the HLA-DM molecules, and then move to the cell membrane (Wolf and Ploegh, 1995).

It has been reported that class II MHC molecules can bind some protein molecules, like sperm whale myoglobin, without any processing other than an extracellular denaturation (Sette et al., 1989a; Streicher et al., 1984), and some T-cells can directly recognize non-processed protein Ags, e.g. fibrinogen, directly presented on APC (Lee et al., 1988). These unusual observations finally found a theoretic explanation after unveiling of the crystal structure of MHC class II molecule (Fremont et al., 1996). The structural study shows that the peptide-binding groove of MHC class II molecule has open ends, which may allow MHC class II molecule to bind to some large peptide fragments. Based on this property of the class II MHC molecule, "MHC-guided Ag processing" was proposed. In this view, after encountering the proteases within the endosomes, protein Ags are unfolded by destruction of disulfide bonds, and then partially digested to generate large protein fragments. Loading of the large protein fragments into the MHC peptide-binding groove occurs within MIIC compartments. T-cell determinants within the fragments are protected from enzymatic digestion after binding to MHC molecules, but the flanking regions are further trimmed by the proteases within the MIIC to generate minimal T-cell determinants (Germain,

1994; Madden, 1995). Generation of an insulin T-cell epitope was monitored in vitro by Delovitch's group in order to address the MHC-guided processing (Lang et al., 1996). The data showed that binding of a large insulin fragment to IA^d molecules was essential for presentation of the insulin T-cell epitope, whereas, in the absence of the MHC molecules, the insulin fragment degraded without generation of the T-cell epitope.

1.3.1.3. APC diversity and Ag processing

Different lineages of APC may differ in their processing machinery, such as the intracellular proteases and their localization, levels of expression of MHC and costimulation molecules, chaperone molecules and sensitivity to environmental stimuli such as cytokines and infections (Manca et al., 1998; Vidard et al., 1992). Thus, presentation of dominant peptides may vary among the different APC populations. Even in the same APC population, peptide presentation may be diverse depending on the routes of Ag internalization, the stage of APC maturation and the tissue localization (Harding, 1996).

Dendritic cells (DC)

DC are considered the most efficient APC population due to their high expression of MHC and co-stimulatory molecules, their vigorous rate of endocytosis and phagocytosis, and mostly, their capacity to activate naïve T-cells (Guery and Adorini, 1995). It has been suggested that DCs represent the natural

adjuvant for T-cell activation (Cella et al., 1997). DCs are bone-marrow-derived monouclear cells, and have very high level of fluid phase pinocytosis, which allow them to concentrate large volumes of macromolecules. Also, it has been shown that DC localized in non-lymphoid tissues are in an immature stage, and characterized with high capacity of Ag capturing and processing, but low capacity of T-cell stimulation (Steinman, 1991). After activation by inflammatory mediators, these immature DCs migrate into the draining lymphoid tissues and become mature DCs. There, they lose the ability to capture Ag, but gain the capacity to stimulate T-cells. Different subclasses of DC have been recently identified (Rissoan et al., 1999). It would be interesting to compare their properties in Ag processing and presentation.

In addition, DC express high amounts of the low affinity type II Fc-gamma receptor (FcγR) and mannose receptors that allow efficient capture of immune complexes and manosylated Ags, respectively (Sallusto et al., 1995). Unlike FcγR, which strongly associates with its ligands, mannose receptor dissociates from its bound Ag quickly following Ag internalization (Stahl, 1992). Therefore, the mannose receptor may only mediate Ag uptake, but the FcγR, like the mIg, may mediate Ag uptake and also affect Ag processing.

Macrophages

Macrophages are active in phagocytosis, and thus, efficient in presentation of particular Ags. Similarly to DCs, the Ag-processing capacity of macrophages is

affected by their stage of maturation, activation status, localization and environmental stimuli (Schneider and Sercarz, 1997). Macrophages may contain different subtypes with various capacities in Ag processing since different macrophage clones with similar phenotype can differentially process protein Ag to generate T-cell epitopes, suggesting that these clones may vary in their proteases such as cathepsins (Rodriguez and Diment, 1992; Walker, 1989). Similar results have been reported by using c-Myc-transformed macrophage cell lines (Trannoy et al., 1993), and demonstrating a great heterogeneity in Ag processing and presentation among these transformed macrophage clones. Techniques to isolate functionally different macrophage populations are required to examine macrophage diversity in Ag processing and presentation.

B-cells

Resting small B-cells poorly present protein Ags due to their inefficient Ag uptake by fluid phase pinocytosis (Lassila et al., 1988). The resting B-cells do not express sufficient amounts of co-stimulatory molecules such as B7 and CD40, and thus, cannot activate naïve T-cells albeit the FcγR- and mIg- mediated Ag internalization may be effective in these B-cells (Noelle and Snow, 1990). Furthermore, the lack of co-stimulation following T-B conjugation may cause T-cell anergy and induce tolerance (Schwartz, 1997). However, it has been suggested that activated B-cells efficiently present soluble protein Ags (Vidard et al., 1996; Wagle et al., 1998). B-cell activation requires a primary signal delivered following engagement of mIg with the specific Ag and co-stimulation signals

provided by helper T-cells (Healy and Goodnow, 1998). Cross-linking of BcR or LPS stimulation could activate resting B-cells without T-cell help. By using activated B-cells as APC, Wagle and Vidard demonstrated that activated B-cells were effective in pinocytosis and phagocytosis, and also efficient in presentation of sufficient peptide to activate specific T-cells, although the efficiency and selective presentation were different from that of macrophages (Vidard et al., 1996; Wagle et al., 1998).

Ag presentation in Ag-specific B-cells are different from that in resting B-cells. First, mIg-mediated Ag internalization is 100-1000-fold more efficient than non-specific Ag uptake by resting B-cells (Song et al., 1995). Second, binding of a protein Ag to mIg may partially enhance expression of costimulatory molecules for T-cell activation (Grewal and Flavell, 1996; Noelle and Snow, 1991). Third, binding of Ag to its specific Ab may alter the Ag processing in B-cells (Simitsek et al., 1995). These unique Ag processing properties may characterize Ag-specific B-cells as the key APC for the regulation of an immune response. Due to the difficulty of isolating large number of naïve Ag-specific B-cell clones, the study of Ag processing pathways in Ag-specific B-cells is hampered.

1.3.2. Circulating T-cell repertoire is auto-reactive

1.3.2.1. Positive and negative selection in thymus

Positive and negative selection is an active process that occurs in the thymus following conjugation of double positive (DP) thymocytes with the thymic APC or epithelial cells. Positive selection rescues MHC-restricted thymocytes. Negative selection deletes auto-reactive thymocytes (Marrack and Kappler, 1997). It is the avidity and affinity between the thymocytes and the thymic APC that determines the fate of the immature thymocytes, either positively selected to become mature and enter the peripheral blood or negatively depleted by apoptosis (Sebzda et al., 1999).

Currently, there are two models explaining thymic selection. The affinity model focuses on the single TcR-peptide-MHC interaction. Different peptide-MHC complexes may provide different affinities to the TcR. The avidity model focuses on the T-cell-APC conjugate, in which the amount of TcR-peptide-MHC complexes formed between the T-cell and APC may be crucial for the T-cell's fate. These two models agree at the molecular level in that a strong TcR signal generated by a high affinity TcR-peptide-MHC interaction and/or many TcR-peptide-MHC ligations may result in a negative selection, and a weak TcR signaling generated by a low affinity TcR-peptide-MHC interaction and/or few TcR-peptide-MHC ligations may lead to a positive selection (Ashton-Rickardt and Tonegawa, 1994; Jameson et al., 1995). Without TcR engagement by peptide-MHC complexes, there will be no signal produced for T-cell survival, and thus,

the immature T-cells will die through a default pathway, programmed cell death (PCD) (Alam et al., 1996; Sebzda et al., 1994).

1.3.2.2. Thymic selection mediated by self Ags

The mature T-cell repertoire is normally shaped by self-protein molecules presented on thymic APC. Previously, it was suggested that common auto-Ags such as myelin basic protein (MBP), proteolipid protein (PLP), Tg and acetylcholine receptor (AchR) etc occurred only outside the thymus, but recent evidence supports the view that most of these auto-Ags are expressed intrathymically (Anderson et al., 2000; Grima et al., 1992; Heath et al., 1996; Pribyl et al., 1993; Wheatley et al., 1992). The medullary epithelium is considered to be highly heterogeneous consisting of a diverse array of different cell lineages and able to generate a mosaic of peripheral self-proteins (Farr and Rudensky, 1998). Alternatively, peripheral APC may internalize tissue-specific Ags and then circulate into the thymus to mediate central tolerance. In this way, tolerance to peripheral, tissue-specific self-proteins is maintained. Since the mature T-cell repertoire is being selected by weak interactions between thymocytes and the thymic-expressing self-proteins, the mature T-cells may retain their weak reactivity to the selecting molecules in the periphery. In fact, it is well accepted that the circulating T-cell repertoire is auto-reactive (Gammon et al., 1991; Lanzavecchia, 1997). It is possible that this weak interaction that mediates thymic

positive selection may also provide signals to maintain the homeostasis of the peripheral mature T-cell repertoire (Sant'Angelo et al., 1997).

1.3.2.3. A novel view of cryptic epitopes of self Ags and thymic selection

For a given self-protein expressed in the thymus, some T-cell epitopes may present dominantly on thymic APC and others may remain cryptic (**Fig. 1.1**). The dominant peptides, expressed at a high density on thymic APC, will provide a high avidity to the APC-T-cell conjugates, and may negatively select the T-cells with a high affinity to the peptide-MHC complexes, and positively select those T-cells with a low affinity to the peptide-MHC complexes. In contrast, the subdominant and/or cryptic peptides, expressed at a low density on the thymic APC, will provide low avidity to the APC-T-cell conjugates, and thus, may positively select high affinity T-cells reactive to the peptide-MHC complexes. Those T-cells binding to the subdominant and cryptic epitopes with very low affinity are eliminated due to insufficient surviving signals generated from TcR-peptide-MHC interaction (Miller and Basten, 1996){Fairchild & Wraith 1992 #1680} (Cibotti et al., 1992). The model shown in **Fig. 1.2** demonstrates how auto-reactive T-cells specific to subdominant and cryptic peptides escape from the thymic selection.

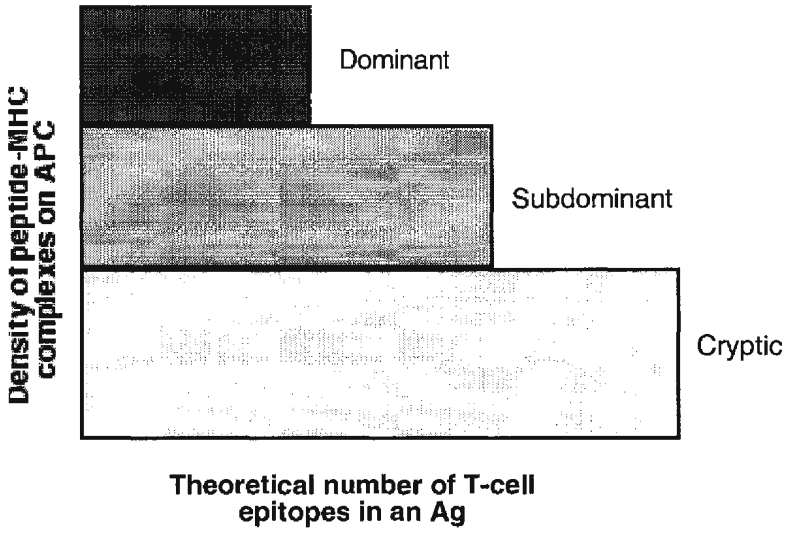

Fig. 1.1. Epitope crypticity in Ag presentation. For a given protein Ag that may contain many potential MHC binding peptides, a hierarchy of peptides may be presented on the APC surface, bound to the MHC molecules, following processing of the protein Ag in a single APC cell. Some of the peptides may be presented in high amounts on the APC surface and denoted as dominant peptides, and others are presented in low amount and denoted as cryptic peptides. Due to the competitive mechanism of the peptide presentation and the influences of the factors affecting Ag processing, only a few peptides present as dominant peptides; most of peptides act as subdominant or cryptic ones.

Cryptic epitopes and thymic selection

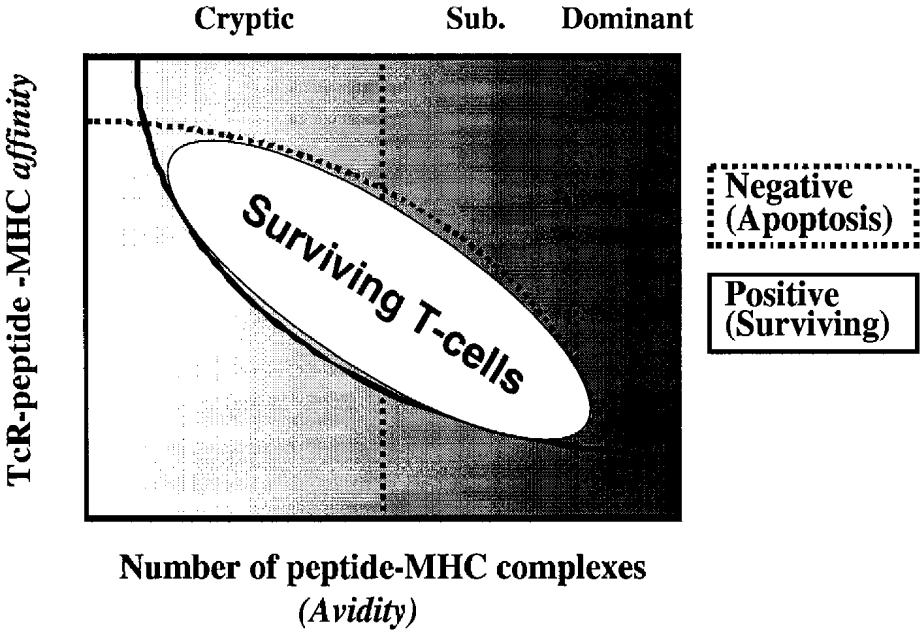

Fig.1.2. Cryptic epitopes and thymic selection. Two parameters determine the fate of an immature thymocyte. One is the affinity (Y-axis) between the TcR and its reactive peptide-MHC complex. The other is the avidity (X-axis) between the T-cell and the peptide-presenting APC, which is mostly dominated by the number of the interacting TcR-peptide-MHC complexes. These two parameters control the strength of interaction between the T-cell and the APC. A minimal threshold (solid curve) of interacting strength is required for delivering a signal for T-cell survival. When the strength is too high and over a certain threshold (dash curve), the apoptotic signals are activated and cause cell death. The first phenomenon leads to positive selection and the second leads to negative selection. The gap between the two thresholds creates the chance for T-cell survival. The concentration of a single peptide present on the APC not only determines the avidity of T-cell-APC interaction, but also determines the dominance or crypticity of the peptide. A dominant peptide provides a high avidity for APC-T-cell conjugates, and a cryptic peptide gives a low avidity. Since the dominant peptides with high avidity may easily activate the apoptotic signals, T-cells reactive to these peptides are mostly negatively selected. Also, because the absolutely cryptic peptides cannot provide surviving signals to T-cells, they do not participate in the T-cell selection. Thus, the T-cell repertoire is established following interaction of TcRs with the dominant, subdominant or some cryptic peptides of the thymic-expressing proteins.

1.3.3. Generation of cryptic T-cell epitopes by receptor-mediated Ag internalization and processing

Unlike particulate Ags, whose uptake in APC occurs through phagocytosis, soluble Ags are mostly captured through surface receptors expressed on the APC, especially in B-cells, in which pinocytosis and phagocytosis occurs at a very low rate (Lanzavecchia, 1990). The most significant receptors are the Fc receptors (FcR) on macrophages or dendritic cells and the membrane Ig (mIg) on B-cells. Other surface receptors such as complement receptors and mannose receptor may play secondary role in Ag internalization by APC (Lanzavecchia, 1990). The receptor-mediated Ag internalization may have two different effects on Ag processing and presentation. First, the receptors can greatly enhance the amount of Ag internalized by APC due to its efficient Ag capture. Second, binding of Ag to the surface receptors may affect the processing of the Ag to generate different T-cell epitopes by APC (Watts, 1997).

1.3.3.1. Enhancing Ag uptake via surface receptors on APC

FcγR

IgG-binding FcR consist of three families, FcγRI, FcγRII and FcγRIII. The FcγRI family has the highest binding affinity for IgG, and is expressed mostly on monocytes and macrophages (Ravetch and Kinet, 1991). The FcγRII family is the

most widely distributed FcR, including FcR on NK cells, neutrophils, platelets, monocytes, macrophages and B-cells. The FcγRII family consists of three subtypes, FcγRIIA, FcγRIIB and FcγRIIC. Two isoforms of FcγRIIB have been reported with different distributions. FcγRIIB1 is the dominant FcR on macrophages, and FcγRIIB2 is expressed only on B-cells (Amigorena et al., 1992). Both isoforms have a higher affinity for IgG1 and IgG2b than IgG2a. FcγRIIA and FcγRIIC are expressed on monocytes and neutrophils, but not on lymphocytes and NK cells. FcγRIIB is the only FcγR restricted to lymphocytes (Ravetch and Kinet, 1991). The FcγRIII family is expressed on macrophages. Unlike FcγR on NK cells, which mediates ADCC, FcγRIII on macrophages may be the binding ligand for immune complexes that trigger macrophage activation (Ravetch and Kinet, 1991). Enhanced Ag uptake mediated by FcγR has been observed both in macrophages and B-cells (Manca et al., 1988; Perkins and Chain, 1986; Stockinger, 1992). Complexing Ag with Ab can increase the capture of Ag-Ab conjugates by APC through surface FcγR, and enhance peptide presentation (Berg et al., 1997). This increased peptide presentation is inhibited in the presence of anti-FcγR mAb.

FcγR-mediated increased Ag uptake may not always boost the immune response against the Ag. First, although increased Ag uptake may occur in resting B-cells through FcγR-mediated Ag internalization, the inactive B-cells cannot activate naïve T-cells without engagement of mIg and may induce T-cell anergy due to the lack of co-stimulation (Schwartz, 1997). Second, in the situation of FcγR-

mediated Ag internalization by Ag-specific B-cells, the Ag-Ab conjugates can cross-link mIg with FcγR and initiate a negative signal to inhibit B-cell activation (Minskoff et al., 1998). The negative signal delivered by FcγR may enable B-cells to regulate the immune response when large amounts of immune complexes (IC) are formed.

mIg

Resting B-cells are not efficient in pinocytosis and have 10-50-fold lower capacity to internalize Ags than that of macrophages (Chesnut et al., 1982a; Chesnut et al., 1982b). Therefore, processing and presentation of non-specific Ags in these B-cells is very inefficient. However, both naïve and memory B-cells express certain amount of mIg, and uptake of specific Ags is 100-1000x more efficient than that of non-specific Ags (Lanzavecchia, 1985). Targeting Ag uptake to B-cells was achieved by conjugating Ag with Abs specific for B-cell surface molecules such as mIg, FcγR and MHC class II molecules (Ozaki and Berzofsky, 1987). Snider et al. addressed Ag internalization of B-cells by conjugating Ag to non-specific Abs, and found that targeting Ag to MHC and mIg, but not the B220 B-cell surface marker, significantly increased Ag uptake and peptide presentation (Snider and Segal, 1987; Snider and Segal, 1989). Interestingly, increased Ag uptake and presentation following conjugation of Ag with FcγRII-specific mAb was observed only in B-cell lymphomas but not in normal B-cells in these studies.

1.3.3.2. Altered Ag processing following conjugation of Ag with membrane receptors

It is clear that receptor-mediated Ag uptake and presentation allows T-cells to recognize Ags at very low concentrations partially due to the efficient Ag internalization (Lanzavecchia, 1990). However, some studies have shown that increased Ag internalization cannot exclusively account for the enhanced Ag presentation (West et al., 1994), suggesting that some post-endocytic regulatory events may be involved in the increased Ag presentation (Amigorena and Bonnerot, 1998). Two mechanisms have been proposed to explain how the post-endocytic events regulate Ag presentation. One is that the surface receptors on APC can target Ag into different endocytic compartments depending on the signals provided by the receptors (Amigorena and Bonnerot, 1998). The other mechanism is that Ag processing can be altered by ligands bound to the Ag, such as FcγR and Ig. The ligands bound to Ags can selectively enhance or suppress presentation of some epitopes within the Ag, depending on the binding area of the ligands within the Ag molecules (Watts, 1997).

In studies of the role of FcR in Ag processing, Amigorena et al. demonstrated that two FcR, FcγRIIb2 and FcγRIII, could differentially mediate Ag processing within a B-cell lymphoma (Amigorena et al., 1998). The B-cells were transfected with different FcRs, and the Ags, HEL and λ receptor, were conjugated with their specific mAbs. Following processing of the ICs within the B-cells, 4 out of 11

epitopes tested were efficiently presented when the B-cells were transfected with both FcγRIIb2 and FcγRIII, but the other 7 epitopes were presented only when the B-cells expressed FcγRIII, but not FcγRIIb2. Therefore, the nature of the receptor that mediates Ag internalization determines the selection of epitopes presented on APC. Watts et al. addressed the role of mIg in Ag processing by using EBV-transformed B-cells with different specificity to tetanus toxin (TT) as APC (Simitsek et al., 1995; Watts and Lanzavecchia, 1993). They found that one of the Ig-expressing B-cells could simultaneously enhance the presentation of one epitope by more than 10-fold while strongly suppress the presentation of another epitope of TT. However, another TT-specific B-cell could present both T-cell epitopes efficiently. These studies strongly suggest that Ag processing in B-cells can be modulated with Ag-bound Ig, and presentation of T-cell epitopes can be either boosted or suppressed depending on the fine specificity of the Abs bound to the Ag molecules. Both studies were performed using B-cells as APC. It would be interesting to examine whether other APC such as macrophages and DC possess similar properties to selectively present T-cell epitopes following Ag internalization through their surface receptors such as FcγR. Manca et al. have shown that Abs facilitate Ag internalization and presentation in macrophages (Manca et al., 1991), but whether Abs can post-endocytically alter Ag processing in these macrophages is unclear.

1.3.4. Ag modulation enhances generation of cryptic T-cell epitopes

1.3.4.1. Modifications within the T-cell determinants

The immune system is programmed to attack foreign pathogens while it remains tolerant to self-proteins. However, post-translational modification of a self-Ag may cause abrogation of the established tolerance to this Ag and thus, trigger autoimmune disease (Mamula et al., 1999). The modification may occur inside or outside the dominant T-cell epitopes of the self-protein. As described above, a Tg peptide containing an iodine-modified structure, T4, has been identified as a pathogenic T-cell epitope, and also Tg-specific T-cell clones only recognize the iodine-modified Tg and T4(2553) peptide (Champion et al., 1991; Dawe et al., 1996). Similarly, Skipper et al. identified a CTL clone that only recognized a modified tyrosinase produced from a melanoma cell (Skipper et al., 1996). The tyrosinase peptide recognized by the CTL clone contained a post-translational conversion of asparagine to aspartic acid, and the conversion had no impact on the capacity of the peptide to bind to the class I MHC molecule, suggesting that antigenic peptides can be derived from proteins following post-translational modification. Also, Meadows et al. found that two CTL clones specific for a H-Y Ag peptide only recognized the modified form of the peptide, which had an additional cysteine residue covalently bound to the cysteine residue at the seventh a.a. position of the peptide (Meadows et al., 1997). The study supported the importance of the post-translational modification in the generation of antigenic determinants.

Post-translational modifications of Ags such as iodination, glycosylation and cystylation have important implications in autoimmunity, since they may commonly occur to some self-Ags. For a given self-Ag, T-cells reactive to the dominant unmodified epitopes within the Ag may be eliminated due to the thymic selection and peripheral tolerance, but T-cells recognizing the modified APL (altered peptide ligand) of the dominant epitopes may escape the thymic selection and tolerance induction. It is unknown what ligands in the thymus mediate positive selection of those T-cells specific for the modified APL.

1.3.4.2. Modifications outside the T-cell determinants

Ag modifications may also occur outside the T-cell determinants within a protein Ag. Such modifications can affect Ag presentation by altering the Ag processing in APC, and thus, generate cryptic peptides. Wood et al. showed that some cryptic T-cell epitopes of an influenza A nucleoprotein could be generated after processing of the N-linked-glycosylated nucleoprotein, but not the non-glycosylated nucleoprotein (Wood and Elliott, 1998). By monitoring the Ag presentation of gliadin, an auto-Ag in celiac disease, Molberg et al. demonstrated that an enzyme, transglutaminase, that catalyses cross-linking between Glu and Lys residues within the gliadin molecules could selectively modify peptide presentation (Molberg et al., 1998). They also found that pathogenic T-cells specific for gliadin reacted strongly to the modified gliadin, suggesting that

transglutaminase-mediated modification of gliadin might be an important trigger for development of autoimmune celiac disease. Griem et al. generated a T-cell clone specific for a cryptic epitope of RNase protein by immunization of animals with metal (Au) ion-modified RNase. The T-cell clone did not recognize native RNase but was reactive to the ion-modified RNase, suggesting that the modification by metal ion was critical for processing RNase to generate the cryptic peptide (Griem and Gleichmann, 1995; Griem et al., 1996). In addition, they found that the T-cell clone also recognized the denatured unmodified RNase, indicating that metal ions may not be required for TcR recognition.

1.3.5. Factors that enhance generation of cryptic epitopes and trigger autoimmune diseases

(summary of autoimmune triggers)

The T-cell activation threshold is determined by the affinity between TcR and peptide-MHC, the avidity between the T-cell and the APC (numbers of TcR-peptide-MHC complexes formed on a single T-cell-APC conjugate) and expression of co-stimulatory molecules (Healy and Goodnow, 1998). Factors affecting activation of auto-reactive T-cells can be divided into two categories. The factors that can enhance Ag internalization and alter Ag processing to generate cryptic epitopes in APC form the first category (**Fig. 1.3**, left). The other category (**Fig. 1.3,** right) includes factors, such as increased expression of TcR and

costimulatory molecules, that can lower the activation threshold of the auto-reactive T-cells and cause recognition of those cryptic epitopes that are marginally presented on normal non-activated APC. Either increased presentation of cryptic epitopes on APC or lowered activation threshold of auto-reactive T-cells may result in activation of auto-reactive T-cells specific for the cryptic epitopes. Synergistic effects between the APC and auto-reactive T-cells may occur in vivo to initiate autoimmune disease.

Activation of autoreactive T-cells specific for cryptic epitopes

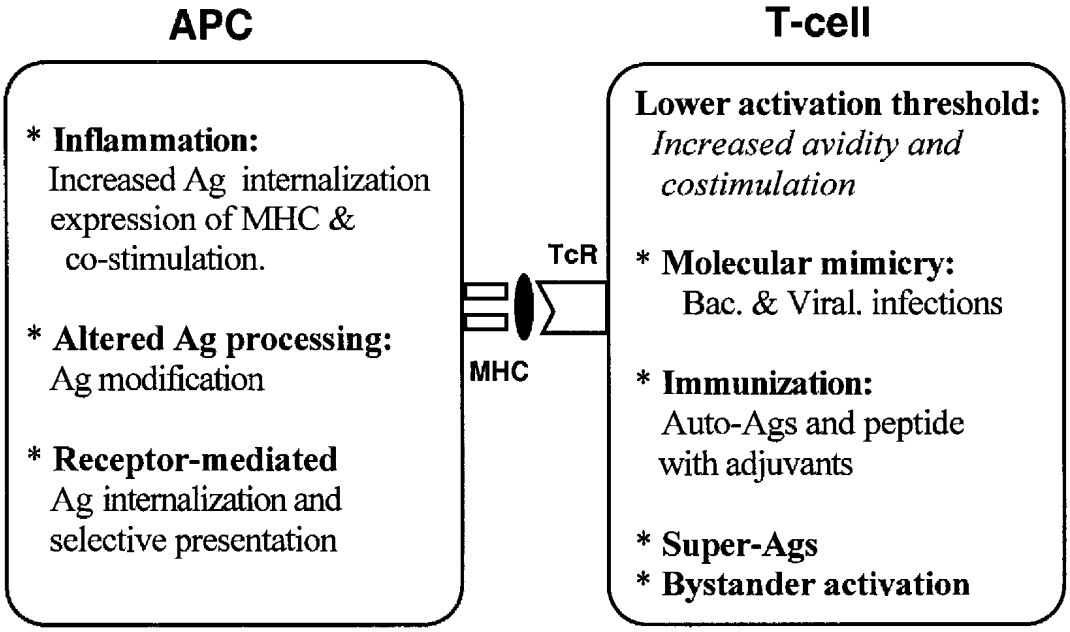

Fig. 1.3. Abrogation of T-cell tolerance against cryptic self epitopes. On the **left**, the diagram shows factors that influence APC function. Inflammation may commonly induce an autoimmune response. In an inflammatory environment, chemokines recruit APC and lymphocytes, and the local accumulation of cytokines may activate the APC to increase expression of MHC, costimulatory molecules and, more importantly, FcR, which, in turn, can facilitate Ag internalization by APC. Activation of APC at the inflammatory site may result in enhanced Ag internalization and presentation and subsequent generation of some cryptic epitopes. In addition, Ag modification can affect processing of auto-Ags in APC and result in a differential presentation of T-cell epitopes. Ligands binding to auto-Ags may also affect Ag processing and selectively enhance or suppress generation of some cryptic epitopes. On the **right**, the diagram shows factors that can decrease the activation threshold of auto-reactive T-cells. Such factors include molecular mimicry, which activates resting auto-reactive T-cells through bacterial and viral infections (microinfections), immunization with self-proteins, non-specific T-cell activation by super-Ags and bystander activation of T-cells. Resting autoreactive T-cells cannot recognize cryptic peptides presented on normal APC in low amounts to become effector T-cells. Following activation, the T-cells express increased amounts of co-stimulatory molecules and high avidity TcR and circulate to the target tissues to initiate autoimmune responses.

1.4. Research rationale and hypotheses

Most of the identified pathogenic Tg peptides have been characterized as non-dominant epitopes due to their insufficient presentation following processing of Tg in vivo and in vitro. Table 1.2 demonstrates that immunization with Tg cannot activate T-cells recognizing T4(2553), Tg2495 and Tg2694 peptides, and peptide-primed LNC cannot be stimulated by Tg in a recall proliferation assay. However, these non-dominant peptides, emulsified in adjuvant, efficiently activate specific T-cells and induce EAT in mice. The described work in this thesis aims to identify conditions or factors that promote the generation of cryptic T-cell epitopes during processing of Tg.

Table 1.2. Cryptic T-cell epitopes of Tg

In vivo immunization	In vitro LNC proliferation against			
	Tg	2495-2511	2549-2560	2695-2713
Tg	+++	-	+	-
2495-2511	-	+++	-	-
2549-2560	-	-	+++	-
2695-2713	-	-	-	+++

Antigen-bound Abs may modulate antigen processing and facilitate generation of subdominant or cryptic T-cell epitopes (Lanzavecchia 1993). A large percentage of patients with HT, and almost invariably all animals developing

EAT have circulating Tg-specific Abs (Weetman, 1994). It remains unclear, if such Abs play a role in promoting the generation of cryptic pathogenic Tg epitopes. We hypothesized that processing of Tg-Ab complexes generates pathogenic but non-dominant T-cell epitopes. The results of this work are presented in chapter 3.

During the course of our experiments, we discovered that a Tg-specific mAb (55H8) was T4-specific and recognized an epitope containing iodine atoms. Also a T-cell hybridoma (3.47) was identified to recognize an iodine-modified epitope within the Tg peptide T4(2553). Based on these observations, we raised the question whether the mAb 55H8 might recognize the bulky iodine atoms of T4(2553) after the peptide was docked within the MHC molecule, leading to a blockade of the peptide-MHC recognition by the TCR of the 3.47 clone. This would provide evidence for an immunoregulatory role of thyroxine-specific mAbs on the development of AT. The results of this work are presented in chapter 4.

Post-translational modification of Ag can trigger autoimmune responses to self-proteins (recently reviewed by Doyle 2001). Metabolic iodination of Tg occurs after its translation and storage in the thyroid gland, and it has been suggested that iodine enrichment of Tg increases its immunogenicity, precipitating an autoimmune response (Rose 1997). We hypothesized that processing of highly iodinated Tg, but not normal Tg, may lead to generation of cryptic pathogenic determinants. The results of this work are presented in chapter 5.

CHAPTER 2

MATERIALS AND METHODS

2.1. Antigens

2.1.1. Purification of thyroglobulin (Tg)

Tg purification was performed following a standard protocol as described before (Chronopoulou 1992). In details, frozen thyroid glands from outbred ICR mice (Bioproducts for Science, Indianapolis, IN) were homogenized in PBS buffer (pH 7.2). The supernatant was centrifuged three times at 16,000g x 10 min. Separation of Tg was performed by passing the clear supernatant through a Sepharose CL-4B column (2.5 x 55 cm) (Pharmacia, Quebec, Canada). As shown in **Fig. 2.1**, the fractions of peak II representing Tg were pooled, concentrated to 3-5 mg /ml PBS with ultra-filtration cells (Amicon, Danvers, MA), and followed by filter-sterilization. The purified Tg was stored at -20^{o}C until use. Tg concentrations were also expressed as molarity of the monomeric form (330 kD, 1 mg/ml = 3.03 μM).

2.1.2. Peptides and synthesis

Thyroid hormone-containing Tg peptides were kind gifts from Dr. Y-C. Kong (Wayne State Univ., Detroit, MI). The preparation of F-moc-L-thyronine, F-moc-L-thyroxine, and the synthesis of T0-or T4-containing peptides was previously described (Kong et al., 1995). The composition and relative purity of each peptide were verified by time-of-flight mass analysis on a Bio-Ion 20 Analyzer (Applied Biosystems Inc., Foster City, CA), and/or by amino acid analysis of peptide hydrolysates (6N HCl, 22 h, 121 ^{o}C) on a Beckman 6300 amino acid analyzer (Beckman, Fullerton, CA). Purity was determined to be >90%. Non-hormone containing Tg peptides and mouse lysozyme peptide were synthesized at more than 80% purity on an Applied Biosystems (Foster city, CA) 430 A synthesizer at the Alberta Peptide Institute. Peptide purity was determined by HPLC and mass spectroscopic analysis. Peptides were blocked with an acetyl group at the N-terminal end, and an amide group at the C-terminal end. The sequences of the peptides used in the studies are listed in **Table 2.1.**

2.2. Antibodies

2.2.1. mAbs and purification

Tg specific mAbs 5D2, 3B3, 2A4, and 3C4 were produced from hybridomas formed by fusing spleen cells from BALB/c mice immunized with human Tg with mouse myeloma NSI/I.Ag 4.1 cells. The binding specificities were characterized previously (Chan et al., 1987). They were purified from culture

supernatants by affinity chromatography on protein G-Sepharose 4 Fast Flow columns (Pharmacia, Baie d'Urfé, Quebec, Canada). The purified mAbs were dialyzed in PBS, concentrated to 1-2 mg/ml, filter-sterilized and stored in PBS at -20 ^{o}C. Tg-specific mAbs 55H8 and 91A1 were derived from human Tg-immunized BALB/c splenic cells fused with the mouse myeloma NSO and was purified from ascites fluid by affinity chromatography. The purified material was concentrated to 1-2 mg/ml in PBS, filter-sterilized and stored in 50% glycerol at -20 ^{o}C until use. Other mAbs were either directly purchased from companies or purified from supernatants of the hybridomas by affinity chromatography on protein G-Sepharose 4 Fast Flow columns. Detailed information on all the mAbs that were used in the studies is provided in **Table 2.2.**

2.2.2. Isotyping of mAbs

Isotyping of mAbs was performed using the ISO-2 kit (Sigma). Briefly, a 96-well micro-titer plate was coated with 100 μl/well of 1 μg/ml of testing mAb in PBS for 1 hour, followed by washing with PBST buffer. Isotype-specific goat anti-mouse Igs provided in the kit were diluted 1:1000 in PBS, and 100 μl of these isotype regents was incubated for 30 min in mAb-coated wells. The plate bound isotype specific goat Ab was detected by peroxidase-conjugated rabbit anti-goat IgG (Sigma A5420) and OPD substrate of peroxidase (Sigma). Development of a brown color in the well indicates positive for the isotype regent added.

2.2.3. Generation of F(ab)'2 fragment of mAbs

Fragmentation of 3C4 to $F(ab')_2$ was done using pre-activated papain as described (Andrew and Titus, 1997). Briefly, papain was pre-activated by cystine in acetate/EDTA buffer for 30 min. Excess cystine was removed by running the papain solution through a Sephadex G-25M PD-10 column (Pharmacia, Uppsala, Sweden). Digestion was performed by mixing 4 mg of 3C4 mAb dialyzed in acetate/EDTA buffer with 0.2 mg pre-activated papain enzyme (Ab : papain = 20:1) in total 3.5 ml of acetate/EDTA buffer. After incubation at 37 °C for 7h, the reaction was stopped by adding 0.3 ml of 0.3 M iodoacetamide. The Fc fraction of 3C4 and/or undigested intact 3C4 was removed by protein-G column. The concentration of $F(ab')_2$ preparation of 3C4 mAb was determined by using Protein Assay Kit (Sigma Diagnostics). The purity of the $F(ab')_2$ preparation was confirmed by SDS-PAGE and its binding to Tg was verified by ELISA.

2.2.4. RIA

Potential cross reactivity for pairs of Tg-specific mAbs was assessed by competitive RIA. Briefly, wells of microtiter PVC plates (Dynatech, Chantilly, VA) were coated by one of the Tg-specific mAbs (3 μg/well) and blocked with 0.3% BSA. Competitive inhibition was performed in the first Tg-specific mAb coated wells by simultaneously adding serial dilutions of a second Tg-specific mAb and fixed amount of ^{125}I-labeled Tg (240,000 cpm/μg/well). After washing

and drying, Tg bound on the first mAb-coated wells was assessed by gamma counting (Wallac).

2.3. Cells and tissue culture

2.3.1. Cells and culture media

The APC cell line TA3, produced by fusion of B cells from CAF_1 mice with the M12.4.1 BALB/c B lymphoma (Allen et al., 1985), was kindly provided by L. Glimcher (Harvard Medical School) and was courteously provided by T. Watts (Univ. of Toronto). TA3 are known to express $A^{k/d}$, $E^{k/d}$, K^dD^d, IgG κ-chain and FcγR molecules while they are negative for IgM, IgD, Thy-1.2 and K^k (Snider and Segal, 1987). The APC cell line LS 102.9 is a B-cell hybridoma (H-$2A^{d,s}$ and H-$2E^d$) obtained from ATCC (Rockville, MD). T-cell hybridomas 5E8 and 6E10 were generated by Dr. V. Rao, a previous student in the lab. The hybrid T-cells are IL-2 secreting, H-2^s –restricted and Tg-peptide specific for TgP1 and TgP2 respectively (Rao and Carayanniotis, 1997).

Cell culture and assays were performed in DMEM medium (Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Bioproducts for Science, Indianapolis, IN, USA), 10 mM HEPES buffer, 2 mM L-glutamine, 100

U/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 5 x 10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO, USA).

2.3.2. Culturing CTLL-2 cells

The IL-2-dependent CTLL-2 line (Gillis and Smith, 1977) was purchased from the American Type Culture Collection (Rockville, MD, USA), and maintained in the above complete DMEM media supplied with 10% FBS and 10% of IL-2 containing supernatant. The IL-2 containing supernatant was prepared by stimulating splenocytes ($5x10^6$ cells/ml) of female outbred rats with 5 μg/ml of concanavalin A for 48h. The supernatant was centrifuged, filter-sterilized and stored at -70 ^oC until use.

2.3.3. Generation peptide-specific T-cell hybridoma

The T-cell hybridoma 3.47 clone was generated by Ms. K. Carayanniotis following a modified method of Perkins and co-workers (Perkins et al., 1991). Briefly, LNC from CBA/J mice, immunized with mouse Tg, were further stimulated in vitro with the same antigen and fused with an TcR$\alpha^-\beta^-$ variant of the BW5147 thymoma (lymphocytes : tumor cells = 1:3) (White et al., 1989) (a kind gift of P. Marrack) using polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN). Selection of hybrid cells were performed by gradually transferring the cells from Hypoxanthine-Aminopterin-Thymidine (HAT) to

Hypoxanthine-Aminopterin (Sigma) and finally to complete DMEM media. Peptide –specific hybridoma cells were cloned by limiting dilution at 0.3% cell/well in the presence of syngeneic RBC as filler cells. Specificity and sensitivity of cloned T-cell hybridoma cells were further tested by T-cell activation assay using TA3 cell line as APC. The 3.47 clone was further characterized to be $CD4^+$, TcR^+ and $I\text{-}A^k$-restricted. They are able to release IL-2 after stimulation with T4(2553) peptide in the presence of TA3 APC.

The 8F9 T-cell hybrid clone was produced by fusing lymphocytes, which were collected from TgP1-immunized B10.BR mice and further stimulated in vitro with the same peptide, with the BW5147 $\alpha^-\beta^-$ thymoma cells. Selecting, screening and cloning were performed similarly as above for generation of the 3.47 clone. The 8F9 T-cell hybridoma is $CD4^+$, TcR^+ and $I\text{-}E^k$-restricted. They release IL-2 after stimulation with TgP1 peptide in the presence of TA3 APC.

2.3.4. Activation assay of T-cell hybridomas

Tg peptide-specific T-cell activation was performed by mixing 10^5 of both T-cell hybridoma and APC in total 200 μl of media in triplicate wells of 96-well flat bottom culture plate. Following 24h culture, 100 μl of supernatant was removed and stored at -20 °C for assessment of IL-2, as measured by the proliferation of the CTLL line and expressed as ^{3}H-thymidine incorporation during the last 6h of total 24h culture.

2.4. Antigen modulation

2.4.1. Formation of Tg-mAb immune complexes (ICs)

Tg-mAb ICs were formed by incubation of equal molar amount of Tg and mAbs for at least 1 h at room temperature. For preparing triplicate ICs mAb-Tg-mAb, equal molar amounts of each component were mixed and incubated for at least 1 h at room temperature. The concentration of the ICs was expressed as molar concentration of Tg in the mixture.

2.4.2. Iodination of Tg by non-radioactive iodine

Iodination was performed in 12 x 75 mm glass tubes coated with 10 μg of IODOGEN (Pierce, Rockford, Illinois). Tg (100 μl of 1 mg/ml per tube) was incubated with 0.25 mM NaI in the IODOGEN-coated tubes at room temperature for 5-30 min. Free iodine was separated from Tg by dialysis in PBS. Iodinated Tg (I-Tg) was concentrated to 3-5 mg/ml by filtration with Centricon 30 micro-concentrator (Amicon, Danvers, MA). The filter-sterilized I-Tg preparation was stored at -20 ^{o}C until use. All the I-Tg used in this study was iodinated for 20 min under the same condition and contained iodine ranging from 60 to 75 atoms per Tg molecule.

2.4.3. Determination of iodine content of Tg

Determination of iodine in Tg samples (16 μg Tg in 0.4 ml 50% glacial acetic acid) was performed as previously described (Palumbo et al., 1982; Saboori et al., 1993). Sodium arsenite was dissolved in 0.15 N sulfuric acid to give a final concentration of 10 mM. Ceric sulfate and ammonium sulfate were dissolved in 2.8 N sulfuric acid resulting in final concentrations of 50 and 150 mM respectively. Bromine was dissolved in double-distilled water as 100 mM stock and diluted to a final concentration of 1 mM bromine prior to the assay. Thyroxin (T_4) was dissolved in 99 vol of absolute methanol and 1 vol of 30% ammonium hydroxide up to a final concentration of 120 μM. For constructing the standard curve 0.4 ml of serial dilutions of T_4 in 50% glacial acetic acid starting at 3840 pmol iodine/400μl (1 M T4 equals 4 M iodine) was mixed with 0.16 ml of concentrated H_2SO_4 in 3 ml cuvettes and incubated for 10 minutes at room temperature. Then, 0.08 ml of 1 mM bromine solution was added and, 15 minutes later, 0.08 ml of ceric ammonium sulfate solution was mixed. Reduction of absorbance at 410 nm over 60 seconds was monitored immediately upon the addition of 0.8 ml of 10mM $NaAsO_2$ into the reaction solution. Iodine content of Tg was expressed as a monomeric Tg molecule.

2.5. Ag processing and presentation

2.5.1. Preparation of APC populations

Splenic B-cells:

Splenic B-cells were enriched by complement-mediated cytolytic elimination of T-cells and accessory cells following a protocol described in Current Protocols in Immunology (Hathcock, 1997). Briefly, RBC lysed (using hypotonic NH_4CL.Tris buffer) splenocyte suspensions (10^7 cells/ml) were incubated together with 20 μg/ml of each mAb: TIB 107 (anti-thy1.2), TIB128 (anti-Mac-1) and TIB227 (anti-DC) for 30 min at 37°C, followed by washing with DMEM medium to remove unbound mAbs. The cytolytic depletion of T-cells and accessory cells was performed by incubating the mAb-coated cells (10^7/ml) in 1:12 dilution of low toxic rabbit serum complement (Serotec, Oxford, UK) for 30 min at 37°C. Surviving cells, mostly B-cells, were isolated with Ficoll-Paque (Pharmacia, Uppsala. Sweden).

Splenic macrophages:

Splenic macrophages were enriched by plastic adherence following a standard protocol (Guery and Adorini, 1995). Briefly, the RBC-depleted splenocytes suspended in DMEM medium (10^7 cells/ml) were incubated in plastic culture petri dishes for 90 min at 37°C with 5% CO_2. Non-adherent cells were discarded and the dishes were gently rinsed with medium. The dishes with adherent cells were subsequently replaced with complete media and incubated at 37°C with 10% CO_2. After 18h culture, the released non-adherent cells mostly DC were

discarded, and adherent cells mostly macrophages were detached with 0.25% trypsin (Gibco) and used for continue study.

Peritoneal macrophages:

Peritoneal macrophages used for this study were thioglycollate stimulated activated macrophages. The mice were injected i.p. with 2.5 ml of 3% thioglycollate solution per mouse. The peritoneal macrophages were harvested 4 days after stimulation.

Dendritic cells:

Dendritic cells were generated from bone marrow progenitor cells following stimulation with GM-CSF (Inaba et al., 1992). Briefly, the bone marrow was prepared from femurs and tibias, and RBC cells in the marrow were lysed with NH_4CL. Fresh GM-CSF (~1000 U/ml) was added every other day to stimulate the adherent marrow cells (10^6 cells/ml/well) in 24-well plate. The proliferating immature DC were harvested by gentle dislodge on day 6, and plated on culture petri dish (10^7 cells/ml) in complete media with GM-CSF. The mature DC were present after 24h incubation and become non-adherent, after which they were collected for further study.

2.5.2. FACS analysis of APC

FACS analysis was performed by incubation of the enriched APC cells with either unlabeled or Biotin-labeled surface maker-specific mAbs. Following mAbs were used: TIB107 (anti-thy1.2), TIB 163 (anti-lyb.8.2), TIB 227 (anti-DC) (ATCC), Biotin-anti-Mac-1, Biotin-anti-B220 (Pharmingen) and Biotin-F4/80 (specific to macrophages) (Serotec, UK). FITC-labeled anti-Ig (Sigma) was used to detect those unlabeled first mAbs, and Streptavidin-FITC (Sigma) was used for labeling the cells bound with Biotin-mAbs. Flow cytometry was performed with a Becton Dickinson FACScan.

2.5.3. Treatment of APC

Fixation of TA3 was performed by suspending 4 x 10^6 cells / ml in 0.05% glutaraldehyde. After 30 sec the reaction was stopped by the addition of an equal volume of 0.2 M glycine.

Mitomycin treatment of splenocytes was performed by incubating RBC-depleted cells ($5x10^7$ cells/ml) with 50 μg/ml of mitomycin (Sigma) at 37°C for 15 min. The treated cells were washed with DMEM to remove the mytomycin completely.

2.5.4. Ag internalization

To monitor uptake of Tg and ICs by TA3 APC, Tg was ^{125}I-labelled using Iodogen (Pierce, Rockford, IL) and ^{125}I-Na (DuPont Canada, Mississauga, ON) according to the manufacturer's protocol. All free ^{125}I-Na was removed by extensive dialysis prior to IC formation and incubation with the APC. The ^{125}I-labeled Tg (170,000 com/μg) was used to pulse the TA3 APC in the form of either ICs with mAbs or alone. The internalized Tg by TA3 cells was measured by counting radioactivity of the cell pellet, and expressed as cpm.

2.6. Evaluation of immune responses against Tg and its peptides

2.6.1. Animals and immunization

Female SJL/J, C3H and CBA mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice at 6-8wk age were immunized s.c. with 100 μg of Tg peptides, Tg or I-Tg emulsified 1:1 in CFA (with *Mycobacterium butyricum*, Difco, Detroit, MI). After 9-12 days, the inguinal, brachial and axillary lymph nodes were collected for in vitro experiments. For induction of EAT, the mice were boosted after 2 wks of the first injection with the same Ag in IFA (Difco). The thyroidal lymphocytic infiltration was examined after 2wks of the second injection.

2.6.2. ELISA

ELISA was performed by coating microtiter PVC plates (Dynatech, Chantilly, VA) with 1 μg/well of Tg or Tg peptides and using an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) as the second antibody. Absorbance at 405 nm was measured using a microplate reader (Vmax, Molecular Devices, U.S.A.).

2.6.3. Cytokine analysis by ELISA

LNCs from mice immunized with 100 μg of Tg or I-Tg in CFA emulsion for 9-12 days were stimulated in vitro with Ags in 200 μl microcultures (4-6 x 10^5 cells/well). Culture supernatants were collected after 48h and stored at -20 °C. IL-2, and IFN-γ released from culture LNCs were determined by Sandwich ELISA. In brief, 96-well microtiter polyvinyl chloride plates were coated overnight with primary capture mAb (2 μg/ml) specific for a particular cytokine and blocked with 0.5% BSA in PBS. Both cytokine standard and LNC culture supernatants were added (100 μl/well). After overnight incubation at 4°C, the secondary biotinylated anti-cytokine mAb (1.0 μg/ml) was used to detect the plate-bound cytokine. Alkaline phosphatase conjugated to Streptavidin and its substrate (Sigma) were used for color development. Absorbance at 405 nm was measured by using a microplate reader (Vmax, Molecular Devices. Menlo Park, CA). The amount of cytokine in each supernatant was extrapolated from the standard curve for the particular cytokine. The primary/secondary anti-cytokine mAbs for IL-2, and IFN-γ are as follow: IL-2: rat anti-mouse IL-2 (Pharmingen) /

Biotin anti-IL-2 (Pharmingen); IL-4: Purified anti-mouse IL-4 from HB188 hybridoma (ATCC) / Biotin-labeled anti-IL-4 (Pharmingen); IFN-γ: Purified anti-mouse IFN-γ mAb from HB170 cell line (ATCC) / Biotin anti-IFN-γ (Pharmingen). Standard IL-2, IL-4 and IFN-γ cytokines were purchased from Pharmingen.

2.6.4. Lymphocyte proliferation assay

Mice were immunized subcutaneously with Tg or I-Tg (100 μg) in complete Freund's adjuvant (CFA) emulsion. After 9-12 days, single cell mixture of inguinal, brachia and axillary lymph nodes was allowed to proliferate for 96 hours against the in vitro Ags in 200 μl microcultures (4-6 x 10^5 cells/well). During the last 24 h, 1 μCi of ^{3}H-thymidine (Dupont, Canada) was added to each culture well in 25 μl of medium. Cell harvesting and counting of the incorporated radioactivity was performed. Stimulation index is defined as: cpm in the presence of Ag / cpm in the absence of Ag.

2.6.5. EAT induction and evaluation

For direct induction of EAT, female 6-8 wk old mice were immunized s.c. with 100 μg Tg or I-Tg emulsified 1:1 in CFA (with *Mycobacterium butyricum*, Difco, Detroit, MI). Two weeks later, the mice were boosted with the same Ag in IFA

(Difco). EAT was assessed by histological examination of the thyroid glands 4wk after the initial immunization.

For induction of EAT by adoptive transfer, donor mice were similarly immunized with Tg or I-Tg in CFA emulsion as direct induction of EAT. After 10 days, the inguinal, brachia and axillary lymph node cells were collected and cultured with 80 μg/ml of respective immunization Ags or OVA for 72 h, followed by adoptive transfer of 10^7 living cells i.p. into each of the syngeneic recipients. Thyroid glands were removed from the recipients after 14 days of adoptive transfer, and fixed in formalin. Thyroid sections were stained with hematoxylin and eosin.

Mononuclear cell infiltration was scored as following: **0.5** = interstitial accumulation of cells between two or three follicles; **1** = one or two foci of cells at least the size of one folicle; **2** = extensive infiltration, 10 - 40 % of the total area; **3** = extensive infiltration, 40 - 80 % of the total area; and **4** = extensive infiltration, > 80 % of the total area.

2.7. TcR V-beta usage of peptide-specific T-cell hybridomas

2.7.1. RNA isolation

Total RNA of T-cell hyridoma 3.47 or 8F9 was extracted by using Trizol reagent (Gibco) according the manufacturer's instruction. Briefly, $5x10^6$ of cells were lysed in 0.5 ml of Trizol reagent. Choloroform (100 μl) was added to separate the cell lysate. After centrifugation, the upper aqueous phase (~250 μl) was collected

for RNA precipitation using isopropanol (250 μl). The isolated RNA was washed with ethanol, dried, dissolved in DEPC-treated ddH_2O and stored at -70 °C.

2.7.2. cDNA synthesis

First strand cDNA synthesis was performed using a cDNA synthesis kit (Pharmacia, Sweden) according to the manufacturer's instruction. About 3-5 μg of RNA was used for cDNA synthesis in a total 33 μl of reaction volume. Not I-d(T)18 primer was used for synthesizing cDNA only from the mRNA. The reaction was run at 37°C for 1 h and terminated by heating at 65 °C for 10 min. The cDNA product was stored at -70°C.

2.7.3. PCR

PCR reaction was performed in a total 100 μl reaction volume containing: 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmoles of each primer (forward and antisense primers), about 200 ng of cDNA temple, 2.5 Unit of Taq DNA polymerase and DEPC-H_2O. All reagents were from Gibco, and the primers for Vβ (Forward) and Cβ (Antisense) (Casanova et al., 1991; Waters et al., 1992) were synthesized in the GSD center for Biomaterials (Toronto, ON). Primer sequences are shown in **Table 2.3**. A 30-cycle amplification was conducted in a Perkin-Elmer DNA Thermal Cycler (Cetus, Norwalk, CT) with 95°C x 1 min, 56°C x 1 min and 72°C x 1 min. The PCR products were analyzed by running the samples on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml).

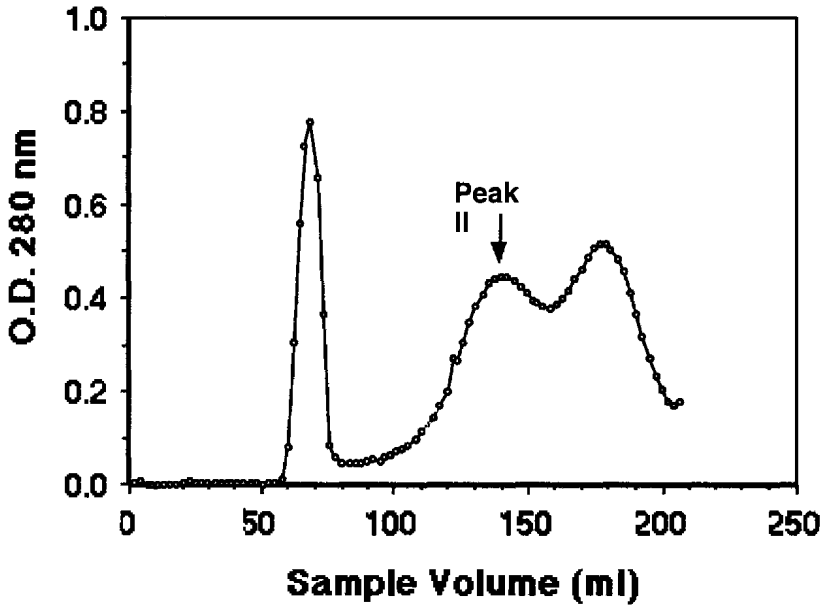

Fig. 2.1. About 100 thyroid glands were homogenized in 2.5 ml ice cold PBS. The homogenate was centrifuged 3x at 16,000g x 10 min at 4°C, and the supernatant was loaded on Sepharose column. Tg was eluted with PBS. Fractions were collected after sample loading. Each fraction or tube contained 2.3 ml eluted solution. Fractions from 47 to 71 (108 to 163 ml of the elution) within peak II were collected as Tg preparation.

Table 2.1. List of peptide used in the studies

Peptide name	Location in Tg	a.a. Sequence
	Hormone-containing Tg peptides (12mers)	
T4(5)	Tg 1-12	NH2-N I F E T4 Q V D A Q P L-NH2
T4(2553)	Tg 2549-2560	NH2-S T D D T4 A S F S R A L-NH2
T0(2553)	Tg 2549-2560	NH2-S T D D T0 A S F S R A L-NH2
T4(2567)	Tg 2559-2570	NH2-A L E N A T R D T4 F I I -NH2
	Non-hormone containing Tg peptides	
TgP1 (17mer)	Tg 2495-2511	Ac-G L I N R A K A V K Q F E E S Q G-NH2
TgP2 (18mer)	Tg 2695-2713	Ac-C S F W S K Y I Q T L K D A D G A K-NH2
	Mouse lysozyme peptide	
ML (16mer)	ML 46-61	Ac-N R G D Q S T D Y G I F Q I N S-NH2

Table 2.2. List of mAbs used in the studies

Name	Hybridoma	Animal	Specificity	Isotype	Source
5D2	5D2	mouse	human Tg	IgG2a	Dr. Shepherd
3C4	3C4	mouse	human Tg	IgG1	Dr. Shepherd
2A4	2A4	mouse	human Tg	IgG1	Dr. Shepherd
3B3	3B3	mouse	human Tg	IgG1	Dr. Shepherd
55H8	55H8	mouse	human Tg	IgG1	Dr. Lymberi
91A1	91A1	mouse	human Tg	IgG1	Dr. Lymberi
T4 clone 5	HB8500	mouse	Thyroxine	IgG1	ATCC
4C5	4C5	mouse	TgP1	-	Our lab
2.4G2	HB 197	rat	mouse FcγR	IgG2b	ATCC
10-3.6.2	TIB 92	mouse	mouse I-A^k	IgG2a	ATCC
14-4-4S	HB 32	mouse	mouse I-E^k	IgG2a	ATCC
M1/70.15.11.5.HL	TIB 128	rat	mouse Mac-1	IgG2b	ATCC
33D1	TIB 227	rat	mouse DC	IgG2b	ATCC
30-H12	TIB 107	rat	mouse Thy1.2	IgG2a	ATCC
RA3-3A1/6.1	TIB 146	rat	mouse B220	IgM	ATCC
Cy34.1.2	TIB 163	mouse	mouse Lyb.8.2	IgG1	ATCC
M2-1C6-4R3	HB 64	mouse	Influenza A	IgG1	ATCC
H16-L10-4R5	HB 65	mouse	Influenza A	IgG2a	ATCC
R4-6A2	HB 170	rat	murine IFN-γ	IgG1	ATCC
11B11	HB 188	rat	murine IL-4	IgG1	ATCC
JES6-1A12		rat	mouse IL-2	IgG2a	PharMingen
JES6-5H4-Biotin		rat	mouse IL-2	IgG2b	PharMingen
BVD6-24G2-Biotin		rat	mouse IL-4	IgG1	PharMingen
XMG1.2-Biotin		rat	mouse IL-4	IgG1	PharMingen

Table 2.3. Oligonucleotides for PCR amplification

TcR Vβ primers*	Sequences (5' – 3')
Vβ 1	CCCAGTCGTTTTATACCTGAATGC
Vβ 2	TCACTGATACGGAGCTGAGGC
Vβ 3	CCTTGCAGCCTAGAAATTCAGTCC
Vβ 4	GCCTCAAGTCGCTTCCAACCTC
Vβ 5.1	GTCCAACAGTTTGATGACTATCAC
Vβ 5.2	AAGGTGGAGAGAGACAAAGGATTC
Vβ 6	CTCTCACTGTGACATCTGCC
Vβ 7	TACAGGGTCTCACGGAAGAAGC
Vβ 8.1	CATTCTGGAGTTGGCTTCCC
Vβ 8.2	CCTCATTCTGGAGTTGGCTACCC
Vβ 8.3	ACGCAAGAAGACTTCTTCCTCCTGC
Vβ 9	TCTCTCTACATTGGCTCTGCAGGC
Vβ 10	ATCAAGTCTGTAGAGCCGGAGGAC
Vβ 11	GCACTCAACTCTGAAGATCCAGAGC
Vβ 12	GAAGATGGTGGGGCTTTCAAGGATC
Vβ 13	AGGCCTAAAGGAACTAACTCCAC
Vβ 14	ACGACCAATTCATCCTAAGCAC
Vβ 15	CCCATCAGTCATCCCAACTTATCC
Vβ 16	CACTCTGAAAATCCAACCCAC
Vβ 17	GAGTAACCCAGACTCCACGA
Vβ 18	CAGCCGGCCAAACCTAACATTCTC

TcR Cβ primer*	Sequences (5' – 3')
Cβ	CCAGAAGGTAGCAGAGACCC

*: TcR Vβ are sense primers. Cβ is anti-sense primers and consensus for Cβ1 and Cβ2 sequences.

CHAPTER 3

ENHANCING AND SUPPRESSIVE EFFECTS OF ANTIBODIES ON PROCESSING OF A PATHOGENIC T-CELL EPITOPES IN THYROGLOBULIN

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Yang Dai performed all the experimental work, data analysis and cloned 8F9 T-cell hybridoma. Karen A. Carayanniotis generated T-cell hybidoma 3.47 and prepares culture media and some of experimental reagents. Petros Eliades and Peggy Lymberi provided mAb 55H8. Philip Shepherd provided mAbs 5D2, 2A4, 3C4, 3B3. Yi-chi M. Kong provided T4(2553) peptide.

3.1. Abstract

Thyroglobulin (Tg) - specific antibodies occur commonly in thyroid disease but it is not clear to what extent they affect Tg processing and presentation to T cells. Here we show that generation of the non dominant pathogenic Tg epitope (2549-2560), containing thyroxine (T4) at position 2553 (T4(2553)), is augmented by Tg-specific IgG mAbs which facilitated FcR-mediated internalization of Tg. However, other mAbs of the same (IgG1) subclass enhanced Tg uptake by APC, but had no effect on the generation of this peptide. Treatment of APC with chloroquine or glutaraldehyde abrogated enhanced generation of T4(2553). The boosting effect was selective since the enhancing mAbs did not facilitate generation of the neighboring cryptic (2495-2511) peptide which is also pathogenic in mice. When Tg was simultaneously complexed to a mAb reactive with T4(2553) and to a mixture of boosting mAbs, the presentation of this epitope was totally suppressed. These results suggest that Tg-specific antibodies alter Tg processing and may boost or suppress the presentation of non-dominant pathogenic determinants during the course of disease.

3.2. Introduction

MHC class II-restricted T cells generally respond to a limited number of immunodominant epitopes in foreign or self antigenic proteins (Sercarz et al., 1993). Antigen-bound Abs are known to influence this response pattern and facilitate generation of subdominant or cryptic T-cell epitopes (Lanzavecchia, 1995) in several ways. First, Abs may increase the efficiency of Ag capture by FcR+ professional APC by 10^3-10^4-fold (Amigorena and Bonnerot, 1998), leading to increased Ag delivery to the processing compartment (Watts, 1997) and production of cryptic epitopes over a critical threshold level required for T-cell activation. Second, Abs may modulate antigen processing by preventing T-cell epitopes from loading onto MHC class II molecules regardless whether these epitopes overlap or not with peptides bound by the Ab combining site (Berzofsky, 1983; Watts and Lanzavecchia, 1993; Ozaki and Berzofsky, 1987). This may suppress the generation of dominant determinants while simultaneously augmenting the presentation of non-dominant epitopes. Third, internalization of immune complexes (ICs) may upregulate MHC class II synthesis or expression of adhesion and costimulatory molecules on APC, increasing their stimulatory capacity. These pleiotropic effects may act synergistically, mediating a spreading of the immune responses to epitopes distinct from those that initiated it. The above concepts are based on experimental evidence obtained mostly with bacterial antigens but they have profound implications in T cell-mediated

autoimmune diseases. Capture of autoantigen by sIg on B cells, or autoantigen-Ab complexes by FcR+ dendritic cells or macrophages, may activate autoreactive T cells (Lin et al., 1991; Mamula et al., 1994) or mediate the spreading of the T-cell response from dominant to cryptic epitopes (Lehmann et al., 1992).

In this study, we have used thyroglobulin (Tg) as a model antigen to examine whether processing of Tg-Ab complexes would generate pathogenic but non-dominant T-cell epitopes. Tg is a 660 kDa homodimer - the largest autoantigen known - that causes experimental autoimmune thyroiditis (EAT), a T cell-mediated disease that is considered as a model for Hashimoto's thyroiditis (HT) in humans (Weetman, 1992). Two observations render Tg an ideal choice for this study. First, none of the known pathogenic T-cell epitopes in Tg has been classified as dominant in EAT studies (Carayanniotis and Rao, 1997). Second, a large percentage of patients with HT, and almost invariably all animals developing EAT have circulating Tg-specific Abs. It remains unclear, if such Abs play a role in promoting the generation of cryptic pathogenic epitopes.

We have developed T-cell hybridoma clones against two pathogenic MHC class II-binding peptides at the C-terminal end of Tg : the subdominant peptide (2549-2560) which is derived following processing of Tg in vivo but not in vitro (Wan et al., 1997; Hutchings et al., 1992); and the cryptic peptide (2495-2511), which is not generated following processing of intact Tg either in vivo or in vitro (Chronopoulou and Carayanniotis, 1992). The aim of the study was to examine if

mAbs bound to Tg would interfere with Tg uptake or processing by APC and promote the generation of these two peptides.

3.3. Results

3.3.1. Characterization of Tg-specific mAbs

Tg-specific mAbs 5D2, 3C4, 3B3 and 2A4 were purified from culture supernatants from respective hybridoma cells. The supernatants were provided from Dr. P. Shepherd. Purification of the mAbs was performed as described in materials and methods by using protein-G-Sepharose column. The 55H8 mAb was provided from Dr. P. Lymberi, and similarly purified from ascites fluid. The binding activities of the mAbs to Tg were confirmed by ELISA (**Fig. 3.1**). The results show that the mAbs raised against human Tg cross-reacted significantly with mouse Tg. Some differences in binding were, however, observed with 5D2 and 55H8 mAbs exhibiting a 10-15-fold better binding to mouse Tg than the others. The subclasses of the mAbs were determined by using isotype-specific Abs. Except 5D2, which is IgG2b, all the other mAbs belong to IgG1 subclass (**Fig. 3.2**).

3.3.2. Uptake of Tg-mAb immune complexes by APC is blocked by an anti - FcR mAb

To directly assess mAb-mediated uptake of Tg by APC, we pulsed TA3 cells for 6 hr with 200 nM of ^{125}I-Tg either in a free form or complexed to various mAbs, and the cell-bound radioactivity was subsequently measured. It was observed that all mAbs significantly enhanced Tg uptake by TA3 and that this effect was abolished in the presence of anti-FcR mAb (**Fig. 3.3**). In preliminary work, all mAbs were shown to exhibit similar binding to native or highly iodinated Tg in ELISA (**Fig. 3.4**).

3.3.3. The 5D2 and 3C4 mAbs boost presentation of the T4(2553) but not the (2495-2511) determinant

To follow the processing of Tg-mAb complexes, we monitored the generation of two neighboring pathogenic peptides T4(2553) and (2495-2511) using TA3 as APC. The first peptide was serendipitously found to activate 3.47, an A^k-restricted, T-cell hybridoma clone which was, nevertheless, unreactive to equimolar concentrations of intact Tg (**Fig. 3.5A**). Processing of Tg bound to 5D2 and 3C4 boosted presentation of the T4(2553) epitope that was now detectable at 5-10 nM, (**Fig. 3.5B**), whereas the 2A4-Tg complex was 10-20-fold less efficient. On the other hand, binding of 3B3 and 55H8 mAbs to Tg had no augmenting effect on the generation of T4(2553), despite their enhancing of Tg uptake by TA3. The enhanced peptide presentation was not correlated with the Ab-mediated Tg-uptake by TA3. For instance, 5D2 and 3C4 mAbs were the most efficient

mAbs that enhanced the peptide presentation, but they did not mediate the highest Tg-uptake; 55H8 and 3B3 mAbs were able to mediate Tg-uptake at same level as the other mAbs, but had no effect on peptide presentation. Also, the boosting effect did not correlate either with the mAb IgG subclass (e.g. 3C4, 3B3 and 55H8 are all IgG1), or with the relative mAb binding to Tg (e.g. 5D2 and 3C4 mAbs showed different reactivity to Tg but augmented the generation of the T4(2553) peptide equally well).

The second epitope (2495-2511) was recognized by 8F9, an E^k-restricted T-cell hybrid clone. 8F9 is similar to 3.47 in its sensitivity for free peptide detection (1-2 nM range) and in its unresponsiveness to intact Tg (2-200 nM range) **(Fig. 3.5C).** However, unlike 3.47, 8F9 did not respond to TA3 co-cultured with any of the above Tg-mAb complexes **(Fig. 3.5D)**. Since 5D2 and 3C4 lowered the threshold for presentation of the T4(2553) peptide without boosting the presentation of the second determinant localized 38 aa upstream in the Tg molecule, the boosting effect of these mAbs could not be attributed solely to enhanced Tg uptake by the complexes. These results suggested that 5D2 and 3C4 exerted a selective augmenting effect by interfering with the loading of the T4(2553) epitope on A^k molecules during Tg processing.

3.3.4. 5D2 and 3C4 mAbs interfere with Tg processing

To exclude the possibility that the 5D2 and 3C4 mAbs exerted signaling effects on TA3 that would generally augment antigen presentation, we titrated Tg or T4(2553) in the presence or absence of a fixed amount of mAb followed by the addition of TA3 and 3.47 T cells. 3.47 was again activated only after processing of Tg-mAb complexes, not intact Tg **(Fig. 3.6A).** In contrast, 5D2 and 3C4 showed no boosting effect on T4(2553) presentation when free peptide was used as antigen **(Fig. 3.6B)** arguing against non-antigen-specific signaling effects of these mAbs on TA3. Addition of chloroquine in the culture abrogated the enhancing effect of 5D2 or 3C4 on the presentation of the T4(2553) peptide following Tg processing, but did not influence the activation of 3.47 cells by TA3 pulsed with free peptide **(Fig. 3.7A)**. Finally, glutaraldehyde-fixed TA3 cells pulsed with Tg-5D2 or Tg-3C4 complexes did not activate the 3.47 clone but retained the capacity to present free T4(2553) peptide to the same T cells (**Fig. 3.7B**). These data indicated that 5D2 and 3C4 exert their effects on Tg processing either by facilitating the generation of the T4(2553) peptide or by augmenting the loading of this epitope on A^k molecules.

3.3.5. FcR-mediated uptake of Tg-Ab complexes is necessary for the generation of T4(2553) peptide in TA3 cells

Following digestion with preactivated papain, we obtained $F(ab')_2$ fragments from the 3C4 mAb. The concentration of the $F(ab')_2$ was determined by Sigma protein determination kit. In ELISA, these $F(ab')_2$ fragments retained Tg-binding

activity similar to that of intact 3C4 (**Fig. 3.8A&B**), and could competitively inhibit 3C4 mAbs binding to Tg (**Fig. 3.8C**). It was subsequently observed, however, that TA3 pulsed with Tg bound to 3C4 $F(ab')_2$ did not activate the 3.47 clone (**Fig. 3.9A**). Also, addition of increasing amounts of FcR-specific mAb in TA3 cultures completely blocked the activation of 3.47 mediated by 5D2-Tg or 3C4-Tg complexes, whereas the same treatment had no effect on the stimulation of 3.47 by free T4(2553) peptide (**Fig. 3.9B**). These results demonstrated that the FcR-mediated uptake of Tg-Ab complexes by TA3 is a necessary step for the formation of the T4(2553) epitope.

3.3.6. The 55H8 mAb suppresses T4(2553) generation during processing of Tg-mAb complexes

We subsequently screened by ELISA all mAbs for potential reactivity against the two Tg peptides. The 55H8 mAb was reactive with the T4(2553) epitope (**Fig. 3.10A**) whereas the 5D2 mAb bound specifically to (2495-2511) (**Fig. 3.10B**). None of the other mAbs reacted with either of these two peptides. This observation explained the lack of augmenting function by 55H8 and further suggested that 55H8 might prevent loading of T4(2553) on A^k molecules during Tg processing possibly due to peptide sequestration, as predicted by the T:B cell reciprocity hypothesis (Chronopoulou and Carayanniotis, 1992; Berzofsky, 1983; Ozaki and Berzofsky, 1987). To test this, equimolar amount of 55H8, Tg and a boosting mAb (3C4 or 5D2) were mixed in wells of microtiter plates in the presence of

TA3 and 3.47 T cells. The 3B3 mAb, which cannot augment presentation of T4(2553) (Figure 3.3.5.B) and does not bind to it (Figure 3.3.11.A), was used as control. It was found that 55H8 abolished the augmenting effects of 3C4 or 5D2 mAbs on the generation of this determinant, whereas the control 3B3 mAb was ineffective **(Fig. 3.11A)**. This dominant suppressive effect was also prevalent when all three potentiating mAbs 5D2, 3C4 and 2A4 were co-cultured with Tg and 55H8 (**Fig. 3.11B**). 55H8 did not interfere with the binding of the enhancing mAbs due to steric hindrance. As shown in **Fig. 3.10C**, none of the available mAbs could inhibit binding of Tg to 55H8 in a competitive inhibition RIA. Similarly, the lack of suppressive effects by 3B3 could not be explained by abrogation of binding due to interference: none of the mAbs inhibited binding of ^{125}I-labeled Tg to 3B3 (**Fig. 3.10D**).

3.4. Discussion

This study demonstrates the presence of Tg-specific Abs that enhance or suppress the generation of a non dominant pathogenic T-cell epitope. Enhancement of peptide presentation results from a combined effect of Abs on increased Tg uptake and altered Tg processing. FcR-mediated endocytosis of IC by APC is a necessary step in this process because of the blocking effects of the FcR-specific mAb and the failure of 3C4 F(ab')$_2$ fragments to augment T4(2553) presentation. On the other hand, increased uptake of IC by APC is not sufficient

to allow generation of the same epitope because the 3B3 or 55H8 mAbs enhanced uptake of IC by TA3 cells but did not augment presentation of the T4(2553) peptide. Altered processing may occur if enhancing mAbs, such as 5D2 and 3C4, remain tightly bound to Tg at the endosomal acidic pH, stabilizing a domain that contains this epitope and facilitating the transport of this determinant in a peptide-loading compartment (Harding, 1996).

The failure of 3C4 to boost generation of the cryptic (2495-2511) peptide provides a clear example of a qualitative effect on Tg processing. This soluble mAb, piggybacked on Tg, enhances Tg capture by TA3 but this does not lower the activation threshold for (2495-2511) to allow its detection by the 8F9 clone. How can the 3C4 mAb that binds to a monomeric Tg subunit of 2748 a.a. residues have such contrasting effects on the generation of two epitopes that are only 38 aa. apart? 3C4 does not bind to (2495-2511) and thus a plausible interpretation would be that endosomal proteases trim the 3C4-bound fragment of Tg and remove the (2495-2511) peptide because it is localized beyond the boundary of 3C4 protection. Thus, it is conceivable that mAbs with different specificities from the ones used here will be found to promote presentation of (2495-2511).

The TA3 line has been produced after fusion of B cells with a B cell lymphoma and its inefficiency in the processing of intact Tg is paradoxical since the rate of fluid phase pinocytosis in B cell tumors is 10-60-fold higher than that of B cells (Chesnut et al., 1982). In earlier studies, Hutchings et al. (Hutchings et al., 1987)

demonstrated that B cells from Tg -primed mice present low amounts of Tg (1 μg/ml) to a T-cell hybridoma clone that was subsequently found to react with (2549-2560) (Champion et al., 1991). Our data and those findings demonstrate that Tg-reactive IgG either in soluble form or as sIg on B cells can mediate enhanced Tg uptake by receptor-mediated endocytosis and modulate Tg processing to enhance the generation of this pathogenic epitope. Interestingly, the above data (Hutchings et al., 1987) also imply that B-cell clones bearing the 55H8 specificity do not occur with high frequency in Tg-primed spleen cells, but this interpretation requires caution since the 55H8 mAb was elicited after challenge with human Tg, whereas Hutchings et al. used a pool of mouse, dog and rat Tg for immunizations.

Other investigators have similarly reported that Abs bound to foreign (Simitsek et al., 1995; Stockinger, 1992; Liu et al., 1996) or self (Berg et al., 1997) antigens can modulate presentation of T-cell determinants. In apparent contrast to our data, Fab fragments did mediate enhanced presentation of peptide antigen in some studies (Simitsek et al., 1995) but the APC were EBV-transformed, tetanus-toxin- specific B cell clones which constitutively internalized IC via their sIg. When B-cell lymphomas were used as APC, use of $F(ab')_2$ fragments in IC did not improve antigen presentation (Stockinger, 1992) and FcR-specific Abs blocked T-cell activation (Stockinger, 1992; Berg et al., 1997), suggesting FcR-mediated augmentation of antigen uptake and presentation. Other FcR-expressing APC such as macrophages or dendritic cells might similarly process

Tg-Ab complexes to generate the peptides studied here but the outcome of the response is difficult to predict given that distinct sets of proteases may be active in different APC (Schneider and Sercarz, 1997). Assays based on adoptive transfer of antigen-specific LNC have clearly shown that the "non dominant" pathogenic (2495-2511) and/or T4(2553) peptides are generated within the mouse (Chronopoulou and Carayanniotis, 1992; Hutchings et al., 1992; Kong et al., 1995) or rat (Balasa and Carayanniotis, 1993) thyroid, possibly via processing of Tg or Tg fragments by intrathyroidal dendritic cells (Voorby et al., 1990).

Antibody-mediated suppression of Tg peptide generation is exemplified by the 55H8 mAb that binds to T4(2553) on Tg and may sequester this epitope from loading onto A^k molecules. This interpretation is in agreement with the T: B-cell reciprocity hypothesis (Berzofsky, 1983; Ozaki and Berzofsky, 1987), according to which, the sIg on B cells, or soluble Ab bound on Ag, can at times negatively steer Ag processing because the part of the Ag within the Ab-combining site is protected from proteolysis and prevented from subsequent loading onto MHC class II molecules. A corollary of this hypothesis would be that soluble 55H8 piggybacked on Tg would exert a dominant suppressive effect on the generation of this pathogenic epitope. This is fully supported by our data : when mixtures of potentiating mAbs and 55H8 were incubated with Tg, suppression was dominant. Suppression could not be attributed to 55H8 blockade of the T4(2553)-A^k complex because the same 5D2-Tg-55H8 IC preparations did not affect presentation of free peptide on TA3 (unpublished data). A reciprocal

relationship is also highlighted by the 5D2 mAb and the (2495-2511)-specific 8F9 clone which recognize overlapping determinants within (2495-2511) : this peptide cannot be generated during processing of 5D2-Tg complexes in TA3 because the T- and B-cell epitopes physically overlap. To our knowledge, our findings provide the first examples that directly support the T: B cell reciprocity hypothesis in autoimmune disease.

The present study reveals an immunoregulatory role for Tg-specific IgG Abs which are quite frequent in HT and include all four subclasses (Weetman and McGregor, 1994; Weetman et al., 1989; Tomer, 1997). The chronicity of the disease favors generation of high affinity IgG1 and IgG4 Abs and it has been suggested that rising titers of high affinity IgG1 anti-Tg may be indicative of impending hypothyroidism (Devey et al., 1989). As extrapolated from our data, such high-affinity autoantibodies may have a neutral, suppressive, or augmenting effect on the generation of pathogenic Tg T-cell determinants, depending on the epitopes they recognize and the genetic background of the individual. In some cases, their net effect could be exacerbation of disease and the spreading of the autoimmune response to non dominant T-cell epitopes (Lehmann et al., 1993). At present, a more direct testing of this hypothesis with Tg-reactive, human $CD4^+$ T-cell clones is not feasible because the epitopes they recognize remain unknown. In addition, we do not know whether Tg-specific, human autoantibodies in HT recognize linear or conformational determinants (Weetman and McGregor, 1994) and where these determinants are precisely localized. Several studies suggest,

however, that autoreactive IgG in the serum of HT patients bind to a limited number of Tg epitopes (Chan et al., 1987; Bresler et al., 1990; Kuppers et al., 1991; Nye et al., 1980; Tomer, 1997) including an immunodominant central region (aa. 1149-1250) (Henry et al., 1992). Autoreactive, Tg-specific Abs may be induced by foreign antigens via molecular mimicry and gradually promote the generation of non dominant pathogenic T-cell epitopes, as suggested in other systems (Lin et al., 1991). Our data are not incompatible with this concept and support the notion that Tg-reactive Abs may play a much more complex role in the regulation of thyroid disease than previously anticipated.

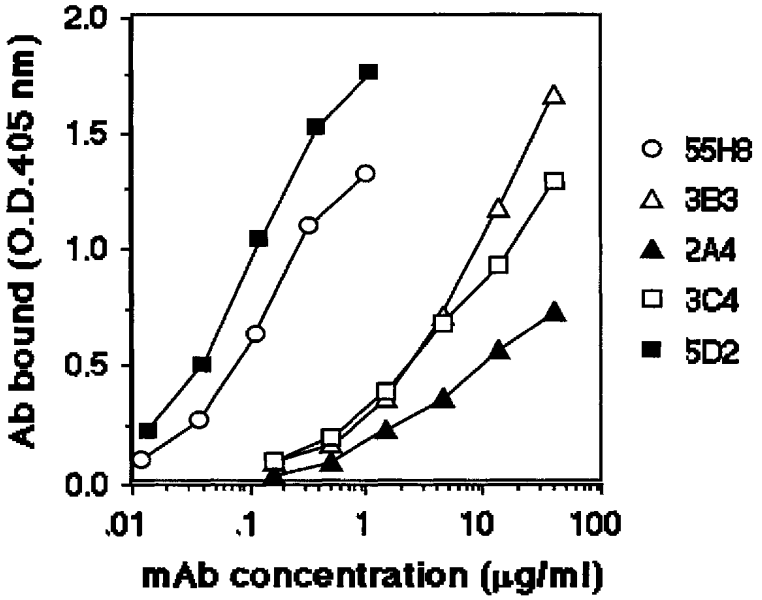

Fig.3.1. Reactivity of Tg-specific mAbs. The 96-well plates were coated with 1µg/well of mouse Tg. Serial dilution of each mAb 55H8, 3B3, 2A4, 3C4 and 5D2 were added, followed with alkaline-phosphatase (AP) -conjugated anti-mouse Ig and AP substrate. The absorbance was measured at 405 nm.

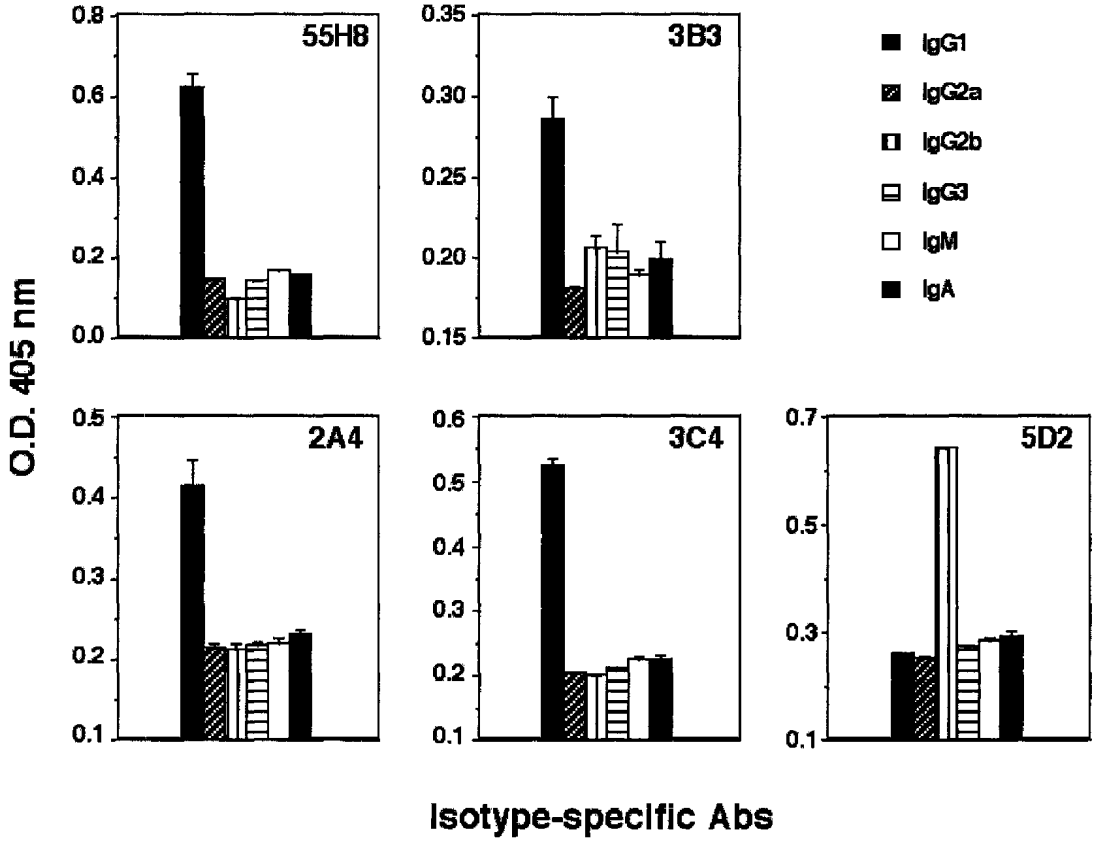

Fig.3.2. Isotyping Tg-specific mAbs 55H8, 3B3, 2A4, 3C4 and 5D2. The 96-well micro-titer plate was coated with 100μl of 1 μg/ml of each testing mAb. Horseradish-peroxidase (HRP) conjugated isotype-specific Abs anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, anti-IgM and anti-IgA were added. The bound isotype-specific Ab was detected by adding OPD-peroxidase substrate. After the appearance of a brown color (about 30-60 min), the reaction was stopped by adding 50 μl 3N NaOH. The absorbance was read at O.D.405 nm.

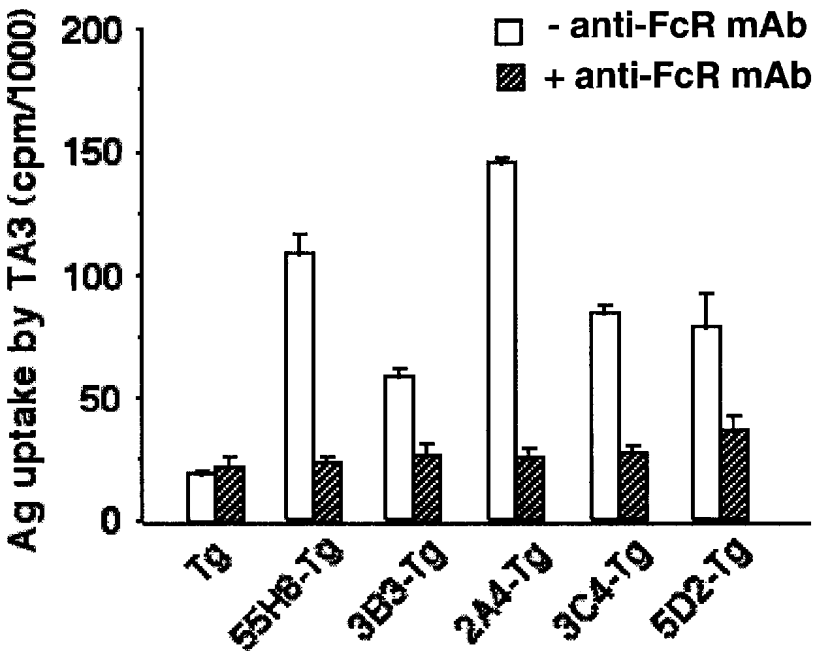

Figure 3.3. Tg-specific mAbs promote IC uptake by APC. Enhanced uptake of Tg-Ab complexes by TA3 is blocked by anti-FcR mAb. ^{125}I-labelled Tg (at a final conc. 200 nM, 170,000 cpm /μg) either free or complexed with equimolar amounts of various mAbs was used to pulse 10^6 TA3 cells in 0.5 ml of culture medium for 6 hr at 37 oC. The anti- FcR mAb 2.4G2 was used at 10 μg/ml. Cell-bound radioactivity was measured after extensive washing. Each value is the mean of triplicate wells. Similar results were obtained in two independent experiments.

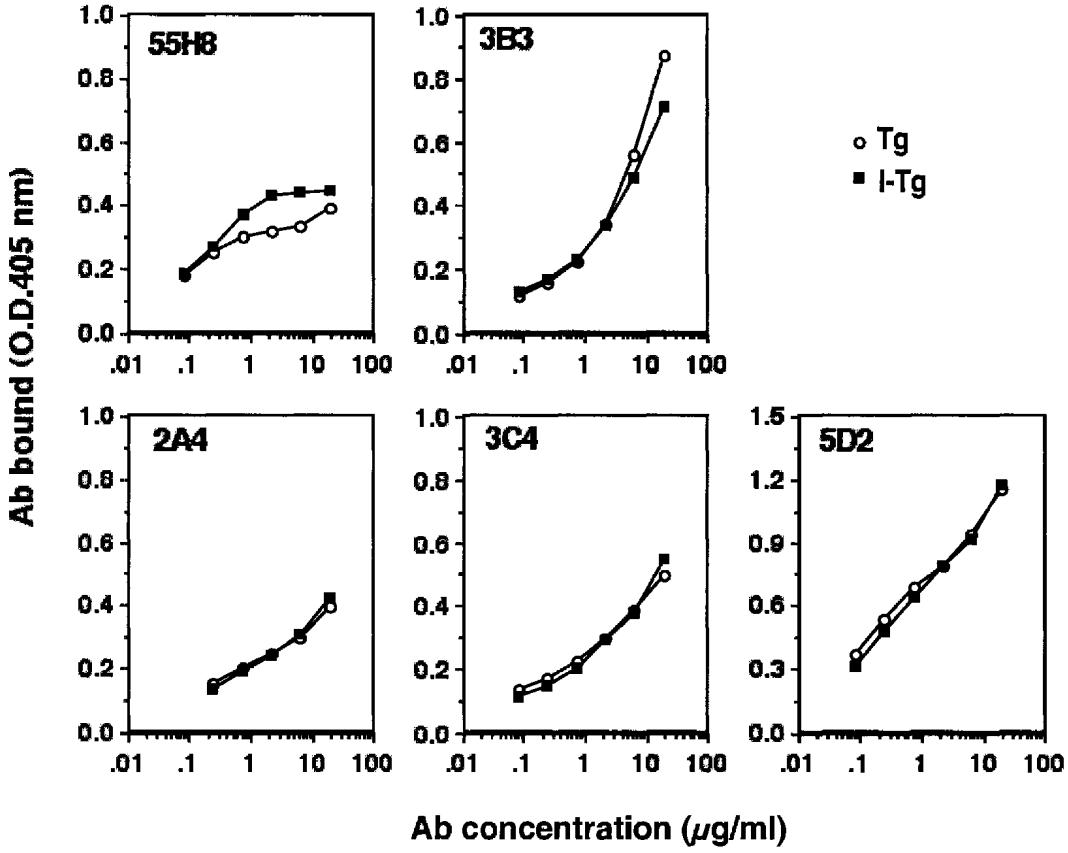

Fig.3.4. Comparison of binding activity of hTg-specific mAbs to mouse Tg and highly iodinated mTg. The microtiter 96-well plates were coated with 1μg of mTg or I-Tg per well. After blocking, serial dilution of Tg-specific mAbs were added. Bound mAbs were detected by second anti-Ig Ab conjugated with AP, followed with AP substrate. The absorbance was read at 405 nm.

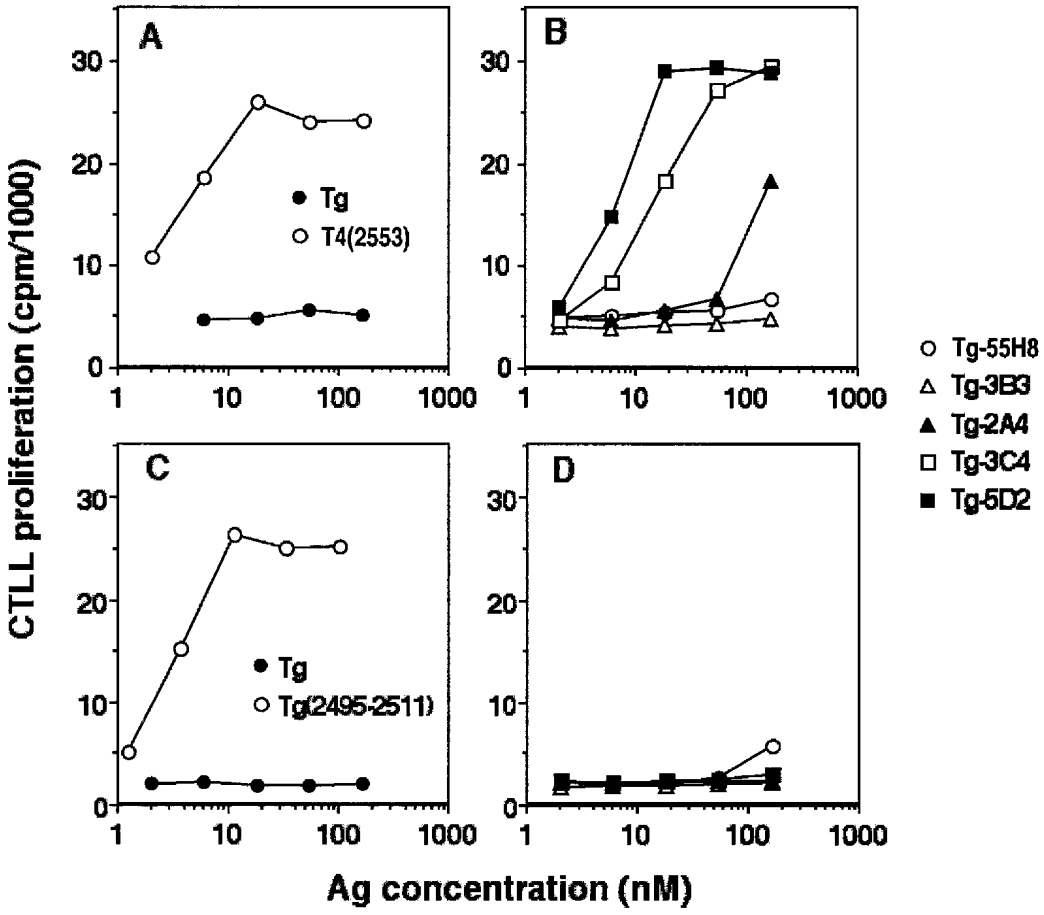

Figure 3.5. Selective generation of the pathogenic Tg epitope T4(2553) following processing of Tg-Ab complexes. IL-2 release following activation of 3.47 (*A* and *B*) or 8F9 (*C* and *D)* T-cell hybrid clones by the antigens shown in the presence of TA3 cells, as assessed by the proliferation of the IL-2-dependent CTLL line. Each point represents the mean of triplicate wells. Similar data were obtained in three independent assays. Neither T-cell hybrid responded to equimolar concentrations of free mAbs (data not shown).

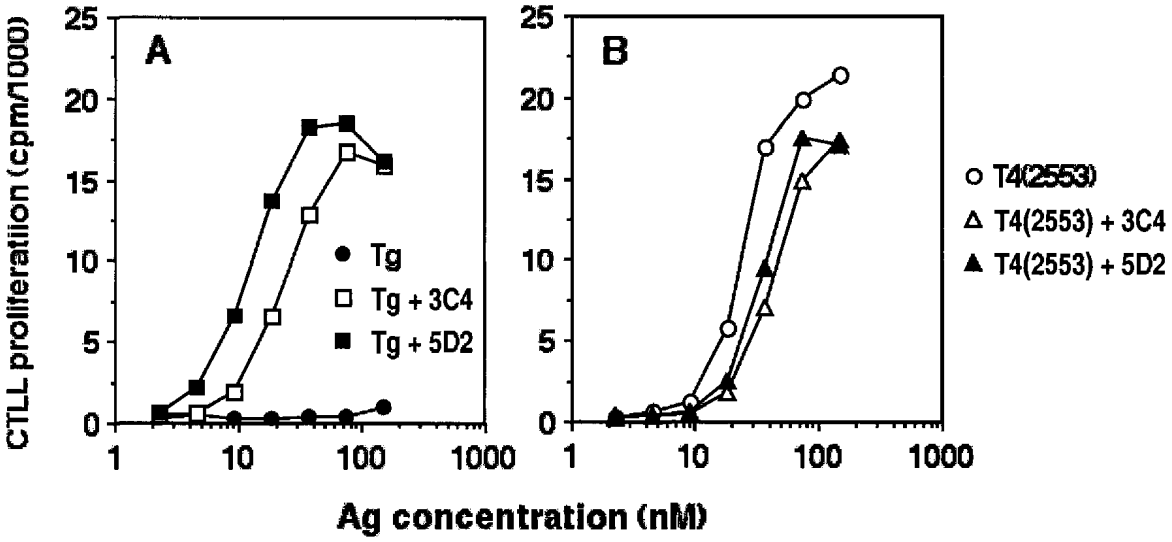

Fig.3.6. Enhanced presentation does not result from a non-specific effect of mAbs on TA3. 3.47 T-cell activation following titration of Tg (*A*) or T4(2553) peptide (*B*) in the presence or absence of a fixed amount (200 nM) 5D2 or 3C4 mAbs and TA3. Each point represents the mean IL-2 release of triplicate wells.

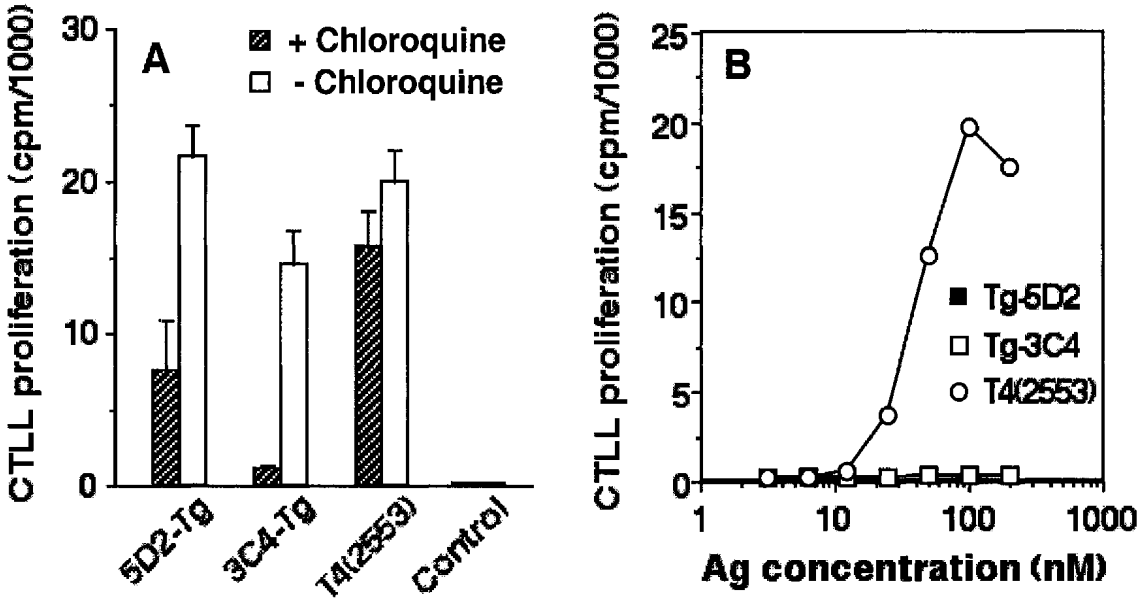

Figure 3.7. Enhanced presentation of T4(2553), mediated by 3C4 and 5D2 bound to Tg, requires an intracellular Tg processing step. Results show the activation of the T4(2553) -specific, IL-2 secreting 3.47 T-cell hybrid clone. *A.* Chloroquine (67 μM) abrogates the enhanced generation of T4(2553) following processing of Ab-bound Tg (50 nM) in TA3 cells, but has no effect on the presentation of equimolar amount of free peptide. *B.* Fixation of TA3 by 0.05% glutaraldehyde abrogates generation of T4(2553) from Tg-Ab complexes but has no effect on the presentation of free peptide.

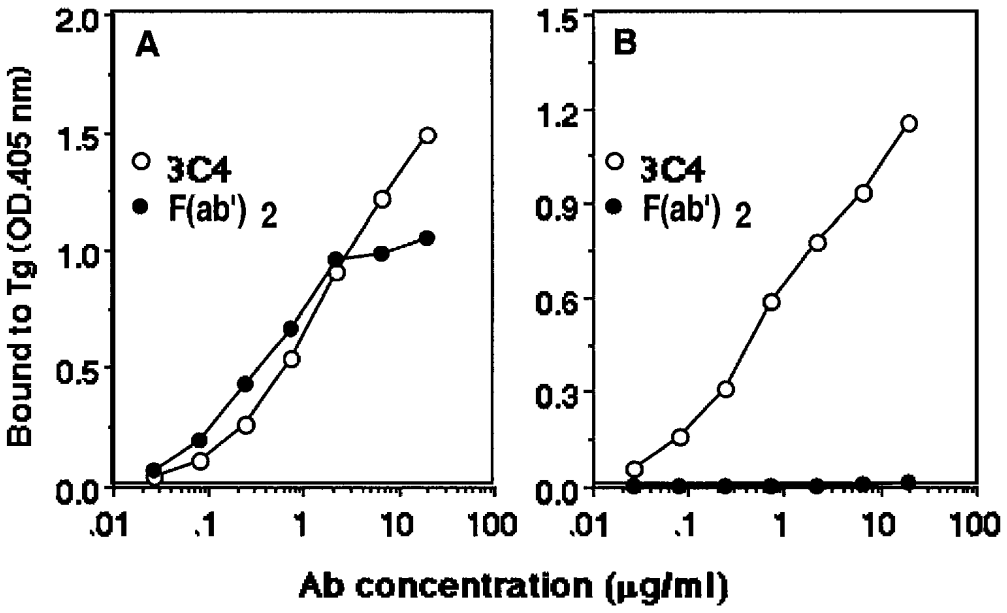

Fig.3.8A&B. The F(ab')2 fragments of 3C4 mAb retained Tg-binding activity similar to that of intact 3C4. The microtiter plate was coated with 1µg/well of Tg. Serial dilution of 3C4 mAb and its F(ab')2 fragments were added to allow binding with Tg. Plate bound mAb or F(ab')2 was detected by anti-Fab (*A*) or anti-Fc (*B*) conjugated with alkaline phosphatase (AP), followed by addition of the AP substrate. The plate was read at 405 nm.

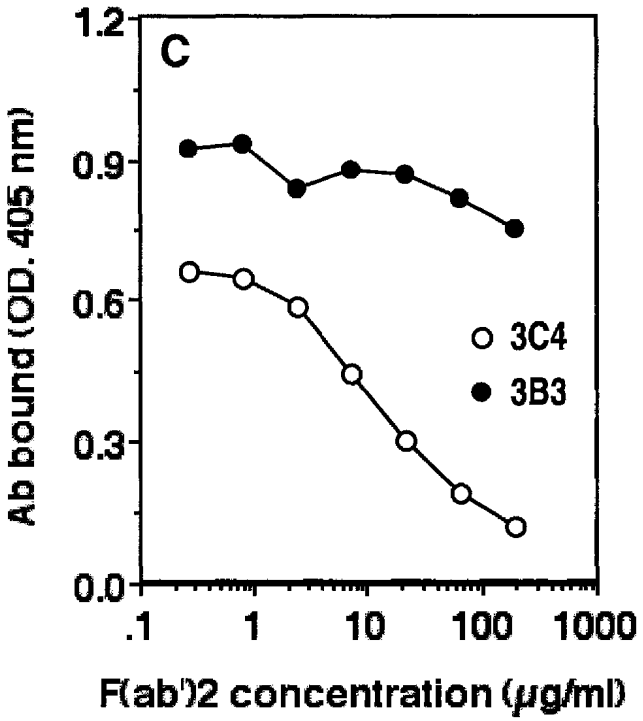

Fig.3.8C. F(ab')2 fragments of 3C4 competitively inhibited 3C4 but not 3B3 binding to Tg. The plate was coated with 1µg/well of Tg. Fixed amounts of 3C4 or 3B3 mAbs (2 µg/ml) were added simultaneously with serial dilution of F(ab')2 fragments of 3C4 mAb. Plate bound mAbs were detected with anti-Fc-AP conjugates and its substrate.

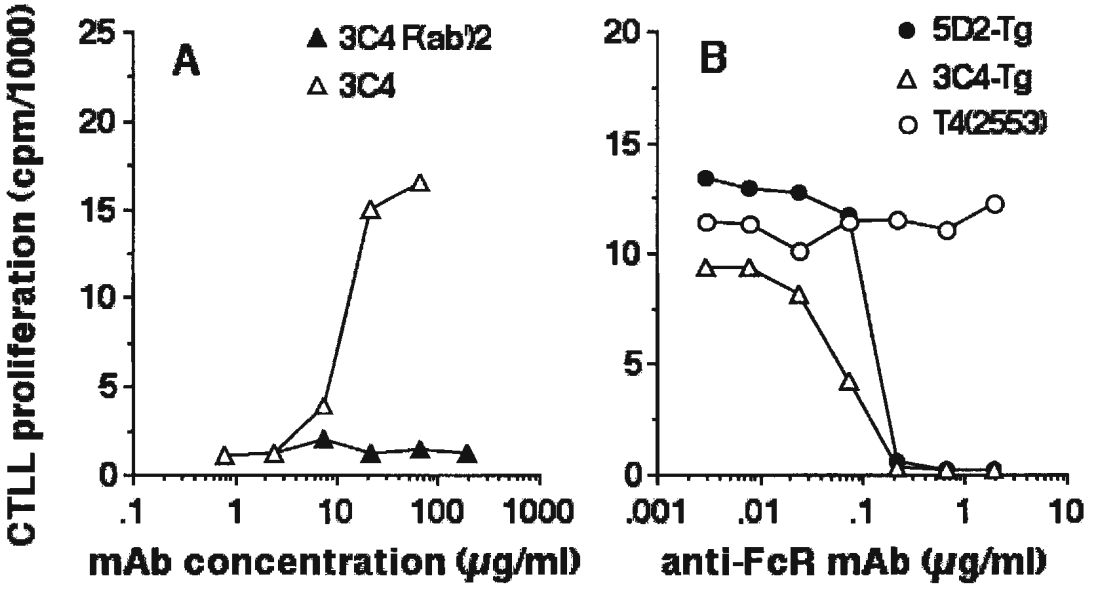

Figure 3.9. FcR-mediated uptake of Tg is necessary for the generation of the T4(2553) epitope. *A.* 3.47 T-cell activation by Tg (180 nM) in the presence of increasing amounts of either intact or F(ab')2 3C4 and TA3 as APC. *B.* Effect of anti-FcR Ab on the activation of 3.47 T-cell with 150 nM of Tg in IC form or equimolar amount of free peptide.

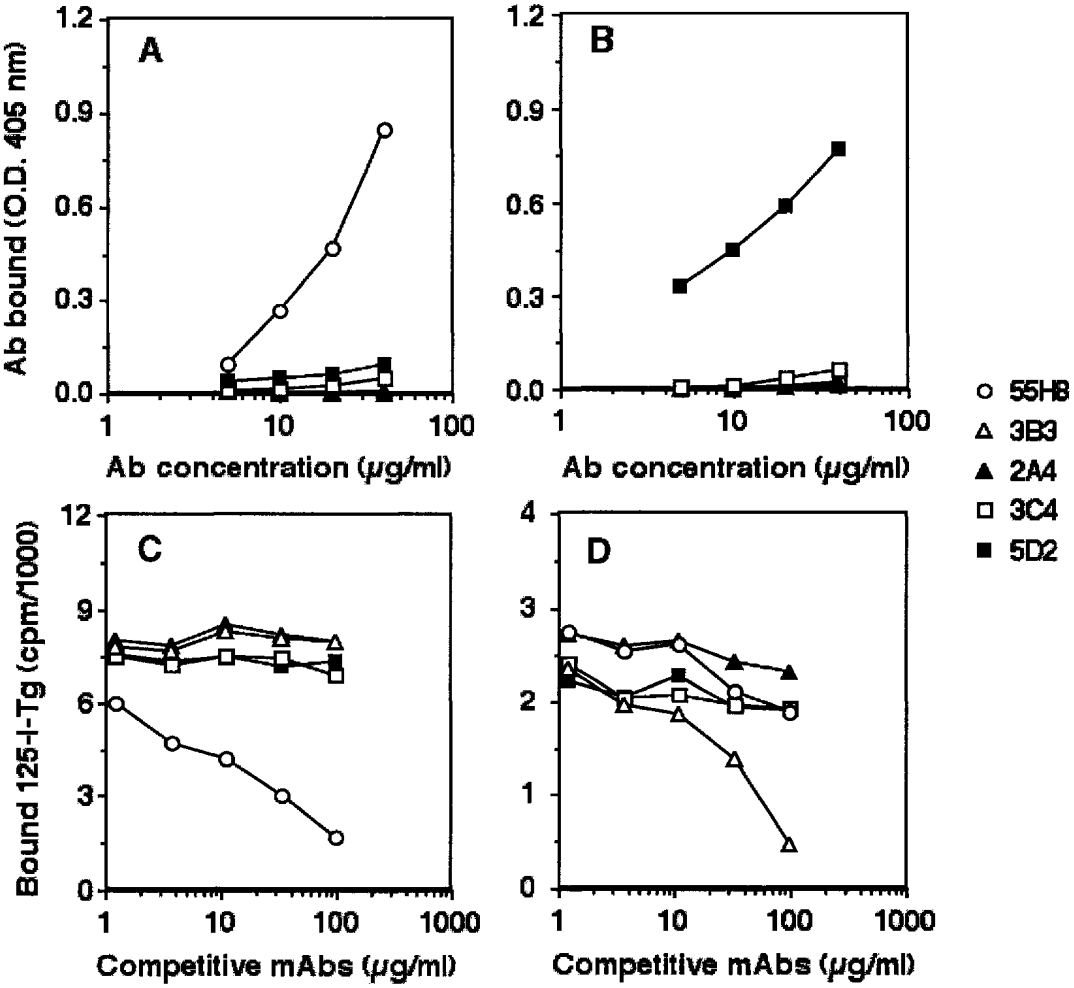

Figure 3.10. Reactivity of Tg-specific mAbs against (A) T4(2553) or (B) (2495-2511) peptides (1 μg/well) as assessed by ELISA. Each point represents the mean value of triplicate wells. *C* and *D* RIA was performed as described in Material and Methods, indicating the competitive inhibition of binding of radiolabelled Tg onto 55H8 (C) or 3B3 (D) mAbs in the presence of the mAbs shown.

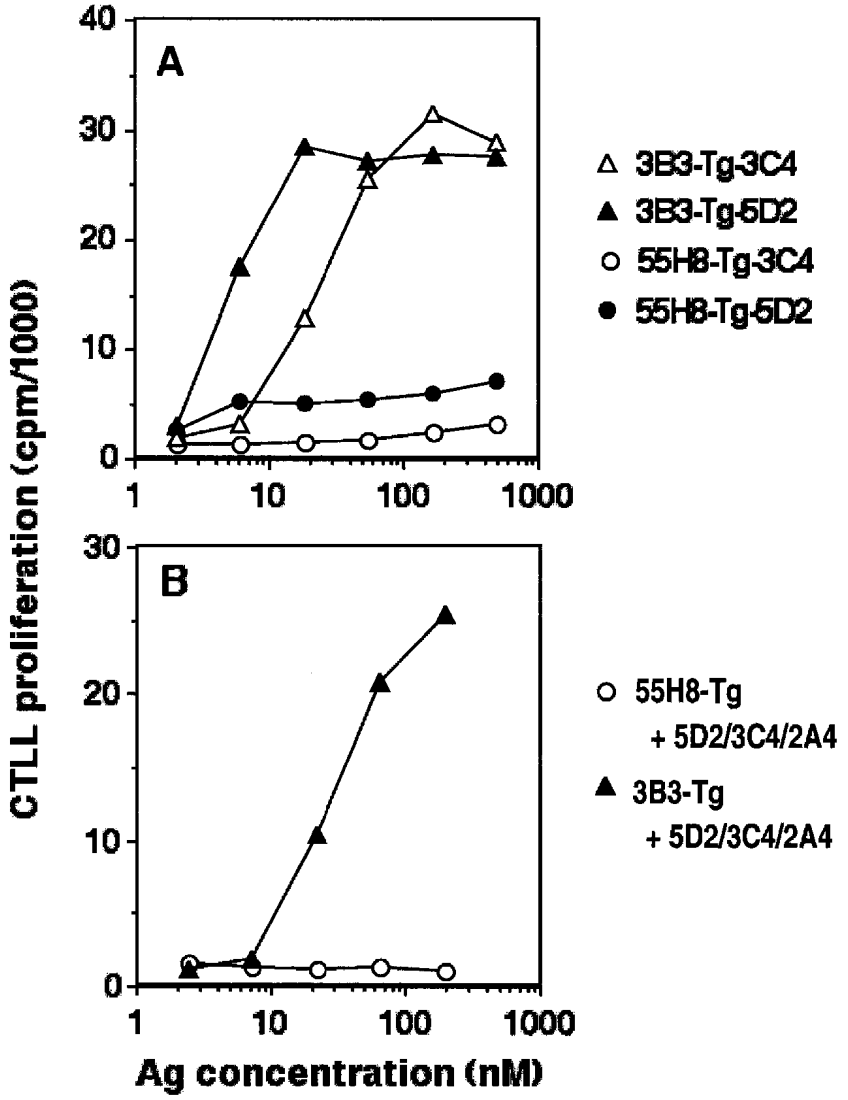

Figure 3.11. 55H8 suppresses generation of T4(2553) during processing of ICs by TA3. *A.* IL-2 release was monitored following 3.47 T-cell activation by Tg mixed at equimolar amounts with 55H8 and either 3C4 or 5D2 mAbs in the presence of TA3 as APC. *B.* 3.47 activation by Tg mixed at equimolar amounts with each of the mAbs shown.

CHAPTER 4

THYROXINE-SPECIFIC ANTIBODIES BLOCK T-CELL RECOGNITION OF A PATHOGENIC THYROGLOBULIN EPITOPE

(Modified manuscript for publication)

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Y. Dai generated 95% of data presented in this manuscript. K. A. Carayanniotis generated 3.47 T-cell hybridoma. P. Eliades and P. Lymberi generated results in Figure 4.10, and provided mAbs 55H8 and 91A1. D. J. McCormick and Y. M. Kong provided peptides T4(5), T4(2553), T0(2553) and T4(2567).

4.1. Abstract

We have generated an A^k-restricted, IL-2-secreting, T cell hybridoma clone (3.47) specific for the pathogenic thyroglobulin (Tg) peptide (2549-2560) that contains thyroxine (T4) at position 2553 [T4(2553)]. The 3.47 clone did not recognize other T4-containing Tg peptides suggesting that presentation of T4 in the context of A^k molecules is not sufficient for activation. On the other hand, the non-iodinated analog peptide T0(2553), which binds to A^k, did not stimulate the 3.47 cells indicating that I atoms within the peptide-MHC complex are necessary for triggering the 3.47 TcR. Following binding of T4(2553) to the A^k groove, T-cell activation was blocked by a Tg-reactive, T4-specific mAb that recognizes the 5' I atom of the outer phenolic ring of T4. In addition, 55H8 inhibited the proliferation of T4(2553)-primed LNC against the peptide-pulsed APC and subsequently transferring of EAT to the recipient mice. This blockade implied sufficient projection of the bulky T4 moiety beyond the MHC groove to enable recognition by Abs. These data demonstrate that some T4-specific Abs can block T-cell recognition of pathogenic epitopes of thyroglobulin and, thus, may exert an immunoregulatory role during the course of thyroid disease.

4.2. Introduction

Thyroglobulin (Tg), (660,000 Dalton), is the only protein known to be iodinated in vivo, and is unique in its ability to couple iodotyrosine residues to form thyroid hormones. Four major hormonogenic sites have been described, corresponding to four tyrosine residues in human Tg at positions 5, 2553, 2567 and 2746 (Rawitch et al., 1983; Malthiery and Lissitzky, 1987). Other tyrosine residues such as Tyr1290 and Tyr685 are also considered as potential sites for thyroid hormonogenesis when the requirement for hormone synthesis increases (Lamas et al., 1989).

Thyroxine (T4) and triiodothyronine (T3) behave as strong haptens, because, in a free form, they do not elicit an anti-hormone response, but become immunogenic only when conjugated with a carrier protein. It is suggested that Tg is the carrier protein for induction of thyroid hormone autoantibodies (THAA) (Benvenga et al., 1987). THAA have had diagnostic use in thyroid diseases (Sakata, 1994), but their pathogenic roles have been rarely discussed. THAA are the most frequently detected auto-Abs in patients with thyroid abnormalities (Erregragui et al., 1996), but they are also prevalent in the normal population (Sakata et al., 1994).

Research on the pathogenic role of the hormonogenic sites within Tg was boosted when a Tg peptide containing a thyroxine (T4) molecule at position of 2553 (T4(2553)) was identified as a thyroiditogenic epitope (Champion et al., 1991; Hutchings et al., 1992). A non-iodinated analog of this T4(2553) peptide could not stimulate a T4(2553)-specific T-cell hybridoma (Dawe et al., 1996). These

studies provided a first direct evidence for the role of iodine in activation of T-cells. However, other studies showed that T-cells recognize T4 and the peptide backbone chain encompassing this hormonogenic site (Kong et al., 1995). Non-iodinated Tg is not immunogenic and cannot induce experimental autoimmune thyroiditis (EAT) (Champion et al., 1987)(Rose et al., 1997). The enhancement of Tg immunogenicity by iodine indicates a potential pathogenic role for hormonogenic sites, since most of iodine incorporated into Tg molecule is utilized for hormone synthesis, albeit other tyrosine residues may also act as iodination sites to form monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues. Formation of T4 and T3 within the Tg may create some new hormone-containing B- and T- cell epitopes and trigger immune responses against Tg.

T-cells recognize peptides presented in the MHC groove, whereas Abs bind their ligands directly. T and B cell epitopes do not usually overlap, but in some cases a BcR may share a similar binding specificity as a TcR. It has been reported that peptide-MHC complexes can be recognized by both TcR and Ab (Stryhn et al., 1996; Aharoni et al., 1991). An Ab binding to the peptide loaded in a class I MHC groove was recently described (Apostolopoulos et al., 1998). Apparently, the bulged middle position of the peptide beyond the class I MHC β-sheet of the binding groove provided enough surface area for recognition by the peptide-specific Ab. However, Abs recognizing peptides lying on the MHC class II binding groove have not been reported. As mentioned above, both T-cells recognizing T4-containing peptide and THAA Abs specific for T4 have been detected in AT. In this study, we examined whether T4-specific Abs can bind T4-

containing peptides presented on class II MHC groove, and thus, block the recognition of the peptide-MHC complex by T-cells.

4.3. Results

4.3.1. Characterization of T4(2553)-specific T-cell hybridoma 3.47

4.3.1.1. The 3.47 T-cell clone is reactive to T4(2553) peptide

T-cell hybridomas were produced following fusion of the LNC from Tg-primed CBA/J mice (H-2^k) with tumor cells BW5147 (TcR $\alpha^-\beta^-$) as described in Materials and Methods. The 3.47 clone was initially selected for its reactivity to mTg-pulsed TA3 cells, but upon continuous culture it lost responsiveness to mTg or intact Tg from human, bovine, porcine or rat origin (**Fig. 4.1A**). However, it was serendipitously found that 3.47 was strongly activated by the conserved Tg peptide (2549-2560) which contains T4 at position 2553 [T4(2553)], while it remained unreactive to two other Tg peptides, (2495-2511) and (2695-2713) (**Fig. 4.1B**).

4.3.1.2. The 3.47 clone is A^k-restricted, $CD4^+$

To examine the MHC restriction of the 3.47 T-cell clone, anti-A^k and anti-E^k mAbs were used to block the activation of 3.47 T-cells. The A^k- and E^k- expressing TA3 cells were used as APC to present T4(2553) peptide to 3.47 T-cells. As shown in **Fig. 4.2A**, as the concentration of anti- A^k mAb increases, the activation of 3.47 clone is significantly inhibited, but the anti-E^k mAb at 100 μg/ml had no effect on 3.47 activation, suggesting the 3.47 T-cell clone is A^k-restricted. Activation of a control T-cell hybridoma 8F9, which is Tg2495-2511 peptide-specific, was blocked by anti-E^k but not anti- A^k mAb (**Fig. 4.2B**), indicating the blocking of the T-cell activation was not due to a non-specific effect. Expression of TcR and CD4 molecules on the 3.47 and 8F9 cells was verified by direct FACS analysis (**Fig. 4.3**).

4.3.1.3. The TcR β-chain sequence of the 3.47 T-cell clone

To identify the Vβ family of the 3.47 TcR, the mRNA was isolated from a fresh culture of 3.47 T-cells, followed by first-strand cDNA synthesis. Primers specific for different Vβ families and Cβ were used for PCR amplification of the unique VDJ region of the 3.47 TcR β-chain. The PCR products were visualized by agarose-gel electrophoresis. A band was obtained when Vβ1 and Cβ2 –specific primers were used in PCR amplification of the 3.47 cDNA, suggesting that the 3.47 TcR uses Vβ1 for β-chain (**Fig. 4.4**). Similarly, the TcR of the Tg2495-2511 peptide-specific T-cell clone 8F9 was identified as using Vβ14. To sequence the VDJ region of the TcR β-chain of the 3.47 clone, the PCR product was extracted

from the agarose-gel and subjected for DNA sequencing with ABI method (**Fig. 4.5**). The sequencing data of the VDJ region of the 3.47 TcR β-chain partially or fully match the mRNA sequences of the mouse TcR Vβ1 gene (Hedrick et al., 1984; Morinaga et al., 1985), the mouse J2.6 gene (Smith et al., 1991) and the mouse TcR Cβ2 gene (Chien et al., 1984; Saito et al., 1984). For the 8F9 T-cell clone, the sequencing data for the VDJ region show that the TcR β-chain uses Vb14 (Malissen et al., 1986; Behlke et al., 1986), Jβ2.5 (Horwitz et al., 1994) and Cβ2. According to the GenBank sequences of the above mouse TcR β-chain genes, the full mRNA sequences for 3.47 and 8F9 TcR β-chains are predicted and shown in **Fig. 4.6A &B**.

4.3.2. The 3.47 clone does not recognize peptides from other hormonogenic sites

To test whether the presence of T4 within any peptide backbone is sufficient to activate the 3.47 clone, we examined the activation properties of two additional T4-containing 12mer peptides of Tg: the T4(5) (a.a. 1-12) and T4(2567) (a.a. 2559-2570). As shown in **Fig. 4.7A**, TA3 APC pulsed with those peptides or free T4 could not stimulate 3.47 to secrete IL-2. Lack of activation was not due to lack of peptide binding to A^k because the T4(2567) peptide inhibited 3.47 activation by the T4(2553) peptide in a competitive inhibition assay to the same extent as the mouse lysozyme peptide (46-62) which is known (Moudgil et al., 1996) to bind strongly to A^k **(Fig. 4.7B)**. The T4(5) peptide and free T4 were not inhibitory suggesting very weak or no binding to A^k. The pathogenic 9mer Tg peptide

(2496-2504) that binds to E^k (Rao et al., 1994) also showed no inhibitory effect. These results confirmed that the backbone amino acid residues within T4-containing peptides were essential for A^k- binding and/or optimal TCR contact that would lead to the activation of the 3.47 clone.

4.3.3. The 3.47 T-cell clone recognizes an iodinated determinant.

Previous observations by Roitt's group had suggested that the (2549-2559) peptide containing de-iodinated thyroxine (thyronine) at position 2553 [T0(2553)] was non stimulatory for a T-cell hybridoma that recognizes the T4-containing analog (Dawe et al., 1996). This was confirmed in the present study since the T0(2553) peptide pulsed on TA3 could not stimulate the 3.47 clone **(Fig. 4.8A)**. In contrast to the findings of that study, however, the T0(2553) peptide could almost completely block the activation of 3.47 by T4(2553) **(Fig. 4.8B)** suggesting strong binding of the thyronine-containing analog to the A^k molecule. The above results clearly demonstrate that the 3.47 clone recognizes a determinant modified by I atoms within the two phenolic-ring structure of T4, and also suggest that the I atoms may not contribute to binding of T4(2553) peptide to the A^k molecule.

4.3.4. I atoms on the phenolic ring of T4 are recognized by T4-specific mAbs

We have previously reported the generation of a Tg-specific mAb (55H8) that binds to the T4(2553) peptide (Dai et al., 1999). We proceeded to examine whether 55H8 is T4-specific by monitoring its reactivity against other T4-containing peptides. It was found that 55H8 reacted similarly against T4(5) and T4(2567) whereas it did not bind to the T0(2553) analog **(Fig. 4.9A)**. These data suggested that the 55H8 mAb is T4-specific and the I atoms on the phenolic ring of T4 are essential for such recognition. This was further confirmed by a competitive inhibition ELISA in which free T4, or T4-containing peptides but not T0(2553) completely inhibited the binding of 55H8 to mouse Tg **(Fig. 4.9B)**.

When we compared the reactivity profile of 55H8 with that of a second T4-specific mAb 91A1, we obtained an overall similar pattern of response with some slight differences. As shown in **Fig. 4.9C**, mAb 91A1 reacted with all the three T4-containing peptides but not T0(2553) peptide on a solid phase despite evidence of a weaker binding to peptides T4(5) and T4(2553). Similarly, in competitive inhibition, all of the T4-containing peptides and free T4 molecule could inhibit binding of 91A1 to mouse Tg (**Fig. 4.9D**) although such inhibition was detectable at higher concentrations of peptides and T4 molecule than those for 55H8. Thus, the two T4-specific mAbs 55H8 and 91A1 possess an overall similar reactive profile to T4-containing peptides.

4.3.5. Different fine specificity of the two T4-specific mAbs

The bulky T4 molecule (763 Dalton) contains 4 iodine atoms, two at positions 3 and 5 of the inner phenolic ring and another two at positions 3′ and 5′ of the outer ring. To examine whether iodine atoms contribute to the recognition sites of T4-specific mAbs, we conjugated rabbit serum albumin (RSA) with T4 (3′, 5′, 3 and 5) or T3 (3′, 3 and 5) and allowed binding of T4-specific mAbs 55H8 and 91A1 to T4-RSA or T3-RSA on ELISA plate. Interestingly, unlike 91A1 mAb, which bound equally well to T4-RSA and T3-RSA, mAb 55H8 bound strongly to T4-RSA but not to T3-RSA **(Fig. 4.10)** indicating that the I atom at the 5' position of the outer ring is an integral component of the determinant recognized by 55H8, but is not recognized by 91A1 mAb. Nevertheless, both mAbs equally bound to mouse Tg.

4.3.6. TcR recognition of the T4(2553) - A^k complex is blocked by the 55H8 mAb

It is well known that peptide-specific Abs do not commonly recognize their ligand within peptide-MHC complexes due to sequestration of the peptide within the MHC groove. Due to the bulkiness of the T4 moiety within T4(2553), and the requirement for T4 in the TCR triggering of the 3.47 clone, we envisaged that the T4-reactive mAbs might bind to the peptide-MHC complex and block peptide recognition by this T-cell clone. To test this, TA3 cells (**Fig. 4.11A**) or peritoneal thioglycollate-stimulated macrophages (**Fig. 4.11B**) were pulsed with 2 μg/ml T4(2553) for 6 hr and were subsequently washed. These peptide-pulsed APC

were subsequently cultured with 3.47 T cells in the presence of increasing amounts of 55H8 and 91A1 mAbs or a control Tg-specific mAb of the same IgG subclass (3B3). Surprisingly, activation of the T-cell hybridoma 3.47 was strongly inhibited by 55H8, whereas the 91A1 and the control mAb 3B3, used at equimolar amounts, had no effect. The blocking effect of 55H8 could not be explained by removal of T4(2553) peptide from the MHC molecule due to a high dissociation rate, because fixation of T4(2553)-pulsed TA3 APC with glutaraldehyde did not affect the blockade of 55H8 on the activation of 3.47 cells (**Fig.4.12A**). The result suggests that some T4-specific Abs exemplified by 55H8 can block recognition of this pathogenic epitope by T cells. An immune complex made by mixing equimolar amounts of Tg and 55H8 could not block activation of 3.47 cells by T4(2553)-pulsed APC, suggesting that free 55H8 is required to block the activation of the 3.47 cells (**Fig.4.12B**).

4.3.7. 55H8 blocks activation of T4(2553)-primed LNC and transfer of EAT

We then proceeded to examine whether the 55H8 mAb could block proliferation of T4(2553)-primed LNC. Since 55H8 binds to free T4(2553) peptide in solution, we could not directly add both peptide and mAb together in LNC cultures. Thus, we first pulsed syngeneic splenic lymphocytes with T4(2553) and cultured the peptide-pulsed splenocytes with 55H8 or 91A1 mAb before mixing them with T4(2553)-primed LNC in a proliferation assay. As shown in **Fig. 4.13**, T4(2553)-pulsed splenocytes could activate T4(2553)-primed LNC with a simulation index of 5.5. When the pulsed splenocytes were treated with 55H8, but not the control

mAb 91A1, prior to mixing with T4(2553)-primed LNC, the proliferation of the T4(2553)-primed LNC was significantly inhibited with a S.I. of 2.1. Furthermore, subsequent adoptive transfer of these in vitro stimulated T4(2553)-primed LNC into syngeneic CBA mice demonstrated that the T4(2553)-primed LNC cultured with the 55H8-treated T4(2553)-pulsed splenocytes could not induce severe EAT in recipient mice showing an average thyroidal lymphocyte infiltration index of 1.42 ± 0.58 (**Fig. 4.14**). In contrast, the T4(2553)-primed LNC cultured with the non-treated or the 91A1-treated, T4(2553)-pulsed, splenocytes induced significantly higher lymphocyte infiltration in the recipient's thyroid glands with an average infiltration index of 2.42 ± 0.58 ($p < 0.05$). The result suggests that 55H8, but not 91A1, could block the activation of T4(2553)-specific T-cells and their differentiation into EAT effector cells.

4.4. Discussion

The capacity of the T4-specific mAb 55H8, but not 91A1, to block stimulation of peptide-specific T-cells by T4(2553)-pulsed APC can be explained by proposing that the bulky T4 molecule projects sufficiently beyond the groove to allow recognition by either T4-specific Ab or specific TcR. In the presence of T4-specific mAb, the activation of peptide-specific T-cells is significantly suppressed due to a steric hindrance.

The T4(2553)-specific T-cell hybridoma 3.47 was generated by fusing BW5147 $\alpha^-\beta^-$ thymoma cells with the Tg-specific lymphocytes from CBA/J mice. The serendipitous finding that 3.47 cells recognize the T4(2553) peptide suggests that the immune response to the T4(2553) epitope may be dominant in CBA/J mouse strain. Other T4-containing Tg peptides, such as T4(5) and T4(2567) , could not activate 3.47 T-cells indicating that presence of T4 in a peptide is not sufficient for 3.47 activation. The amino acid sequence is also essential for the recognition of the T4(2553) peptide by 3.47 cells. The peptide analog T0(2553) containing thyronine instead of T4 at position 2553 did not activate 3.47 T-cells suggesting that the I atoms form part of the epitope recognized by 3.47 TcR. Thus, the T4 molecule in the T4(2553) peptide is necessary, but not sufficient for activation of the 3.47 T-cells. Previous work proposed that the iodine atoms in T4(2553) peptide were essential for loading the peptide into class II MHC molecule (Dawe et al., 1996), because the T0(2553) peptide could not competitively inhibit the activation of the T4(2553)-specific T-cells by T4(2553) peptide. In contrast, we have demonstrated that the T0(2553) peptide can inhibit the activation of 3.47 T-cells suggesting the T0(2553) peptide is able to bind to A^k molecule and I atoms are thus not required for efficient binding of the peptide to MHC. Our data agree with the previous suggestions that T-cell recognition of T4(2553) requires presence of the backbone amino acid residues (Kong et al., 1995). Normal Tg purified from outbred mice may contain T4 at position 2553, since position 2553 is the second important hormonogenic site after the position 5 and accounts for 25 % of hormone synthesis (Dunn and Dunn, 1999). It has been reported that

circulating Tg in healthy individuals is poorly iodinated, but the iodine content of Tg increases in patients with thyroid disorders (Schneider et al., 1983). It is possible that self tolerance is established by Tg with low iodine content. Enhanced iodination and hormone synthesis may break tolerance against Tg and induce autoimmune responses.

The mAb 55H8 and 91A1 were derived from human Tg-immunized BALB/c splenic cells fused with the mouse myeloma NSO. The T4-specificity of the mAbs was examined with various T4-containing peptides and T4(2553) analogs. The difference in the fine-specificity between the 55H8 and 91A1 mAbs was identified using T4- and T3- conjugated RSA. The mAb 91A1 bound to both T4- and T3-RSA, but 55H8 recognized only T4-RSA. The 55H8, but not 91A1 inhibited the activation of 3.47 cells by T4(2553)-pulsed APC indicating binding of 55H8 to T4(2553) presented on A^k MHC. Abs reacting to peptide-MHC complexes have been frequently reported (Stryhn et al., 1996; Murphy et al., 1992; Aharoni et al., 1991; Nygard et al., 1991). Furthermore, an Ab recognizing its specific peptide ligand presented in the MHC groove has been described indicating that some peptides buried in MHC groove may still present sufficient contact surface for Ab binding (Apostolopoulos et al., 1998). T4(2553) is unique in its structure. The bulky T4 molecule accounts for nearly 50% of the total molecular weight of the peptide. The peptide does not fit into all the restricting pockets of the binding motif of A^k molecule (Fremont et al., 1998; Weber et al., 1998), but the presence of aspartic acid at the N-terminal of T4(2553) is the most stringent requirement

for binding to A^k molecule. The amino acid following the aspartic acid (normally tyrosine) is not part of the agretope that binds to A^k molecule implying that it may project out of the groove and participate in TcR recognition. Our results suggest that the T4 molecule within T4(2553) is part of the epitopic moiety for 3.47 T-cell recognition. The bulky T4 molecule is large enough to occupy the paratope of Ab which can accommodate 15-22 amino acid residues with a contacting surface of 650-900 angstroms (Garcia et al., 1992; Harris et al., 1992). Given the structural similarity of the complementarity-determining regions (CDR) between TcR and Ig (Bentley et al., 1995; Fields et al., 1995), the 3.47 TcR, like some anti-T4 mAbs, may not recognize all the iodine atoms present on the T4-phenolic-ring. T4(2553) analogs containing mono-, di- and tri- iodothyronine may address the fine specificity of 3.47 TcR.

Thyroid hormones are haptens. They do not induce immune response unless they are coupled to a carrier protein. It is widely accepted that Tg is the carrier molecule of T4 and T3 for generation of THAA (Erregragui et al., 1996; Sakata et al., 1990). Most of the THAA specifically recognize T3, but other specificities such as T4, T3+T4, reverse T3 +T4 and T3 + reverse T3+ T4 are also frequently observed (Benvenga et al., 1987; Sakata, 1994). As shown in this study, 55H8 Ab binds to T4-RSA, but not to T3-RSA. On the other hand, 91A1 Ab cross-reacts with T3 or T4 –RSA. Whether these different types of THAA play a different role in hormone metabolism or pathogenesis of thyroid diseases is not clear. The occurrence of THAA in patients with thyroid disease and healthy individuals

(Sakata et al., 1994; Bryhni et al., 1996) suggests that some THAA may be harmless, or maybe even protective. The relationship between different types of THAA and thyroid disease should be addressed in future studies. The pathogenetic role of THAA may also be due to an effect on the metabolism of thyroid hormones. It has been suggested, however, most THAA do not affect peripheral T4 and T3 levels due to their lower affinity than thyroxine-binding-globulin (TGB) for thyroid hormones. The existence of THAA mostly reduces hormone disposal (Costante et al., 1990), but extremely high affinity THAA may cause hypothyroidism by competitive binding to peripheral T4 and T3. The importance of THAA affinity in regulating the T4 and T3 level in blood raises the question whether blocking effect of THAA on the activation of T-cells specific for hormonogenic sites is a matter of affinity competition between TcR and Ab as described in this study. Abs such as 55H8 may bind to hormone presented on MHC and thus block TcR recognition. Abs such as 91A1, with lower affinity for T4, may not be able to block the T-cell activation. The peptide binding assay showed that 55H8 bound to T4(2553) better than 91A1. If the affinity of THAA is the key for blocking T-cell activation, generation of Abs with extremely high affinity for T4 may help in suppressing EAT induced by T4-containing peptides.

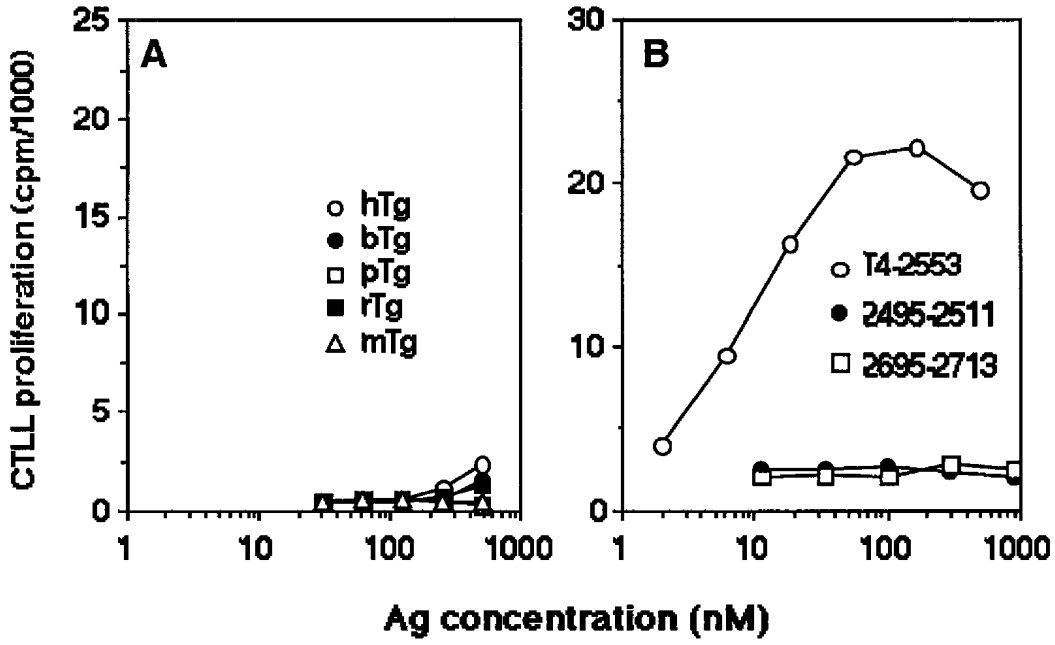

Fig. 4.1. The 3.47 T cell hybridoma clone specifically recognizes the Tg peptide T4(2553). Activation of the 3.47 clone was performed by culturing the 3.47 cells (10^5/well) with an equal number of TA3 APC in the presence of serial dilutions of Tg preparations from various species (**A**) and Tg peptides (**B**) as shown. IL-2 secretion of 3.47 T-cells was monitored by CTLL proliferation. Points are means of triplicate wells. Standard deviations were less than 10% of the means.

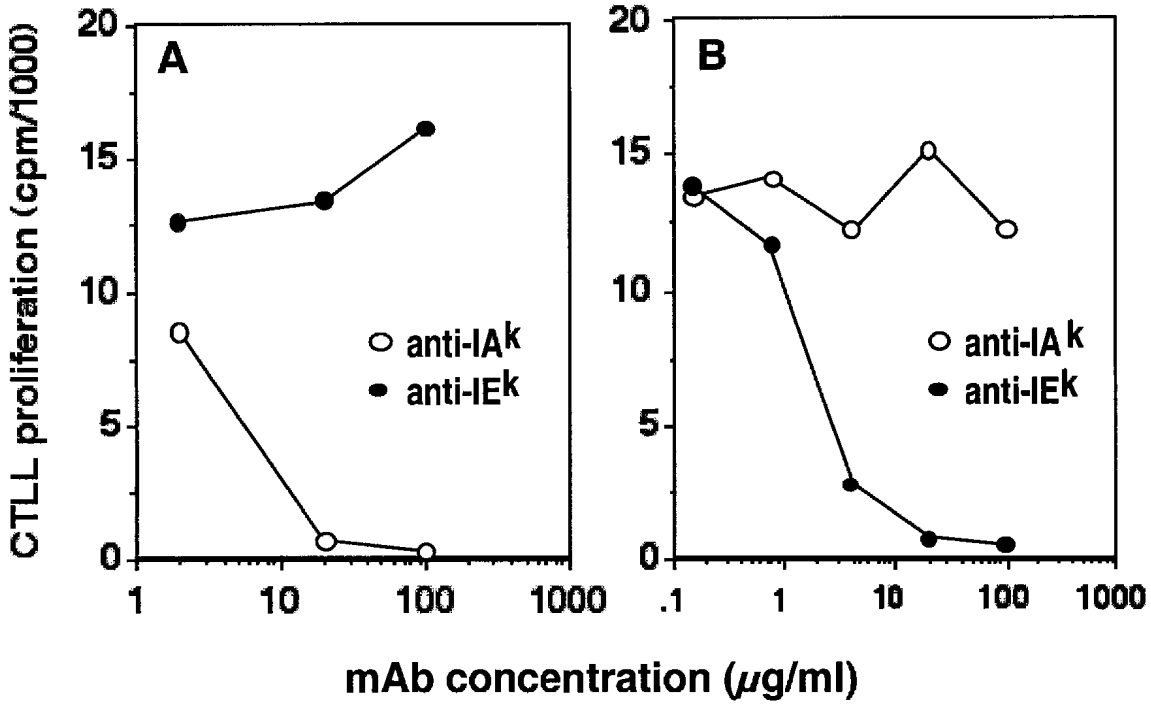

Fig. 4.2. MHC class II restriction of 3.47 and 8F9 T-cell hybridomas. T-cell activation was performed by culturing 3.47 (**A**) and 8F9 (**B**) 10^5 cells / well with an equal number of TA3 APC and a fixed concentration (0.2 μg/ml) of the respective stimulating peptide: T4(2553) (**A**) or Tg2495-2511 (**B**). Serial dilutions of anti-MHC mAbs were added in the culture for blocking. CTLL cell proliferation was followed over 24h culture to monitor the IL-2 secretion in the culture supernatants. Without blocking mAbs, the CTLL proliferation for the stimulated T-cells was: 3.47 = 13,120 cpm; 8F9 = 14,568 cpm.

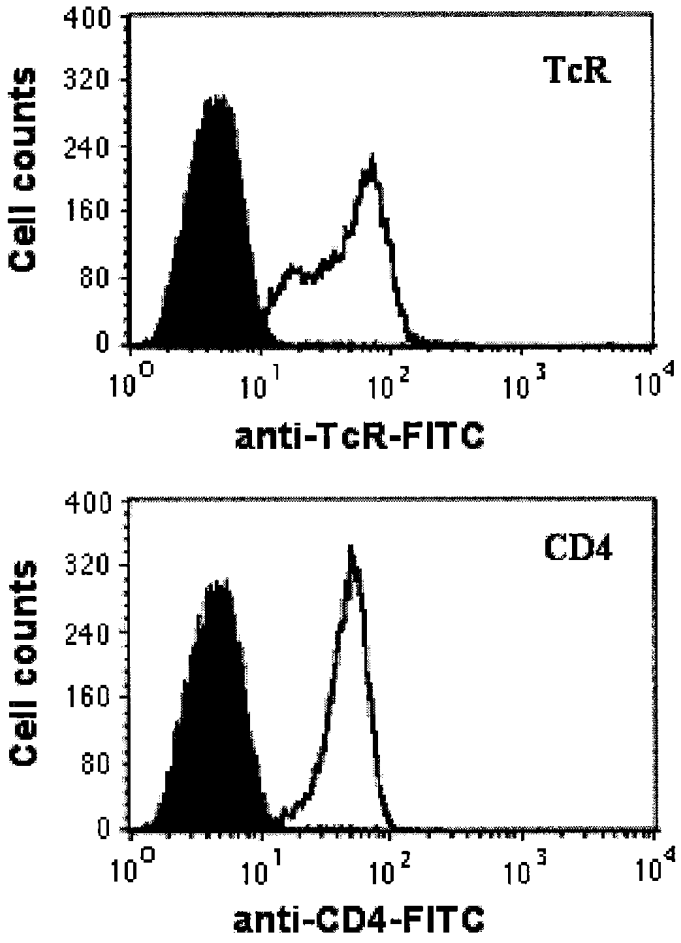

Fig.4.3. TcR and CD4 expression on 3.47 T-cells. 2.5 x 10^5 3.47 cells were directly stained with FITC-labeled anti-mouse TcR or CD4 for 30 min. The results are shown as non-filled curves. As a control, the 3.47 cells were similarly stained with FITC-labeled anti-mouse IgG (Fc) (Filled).

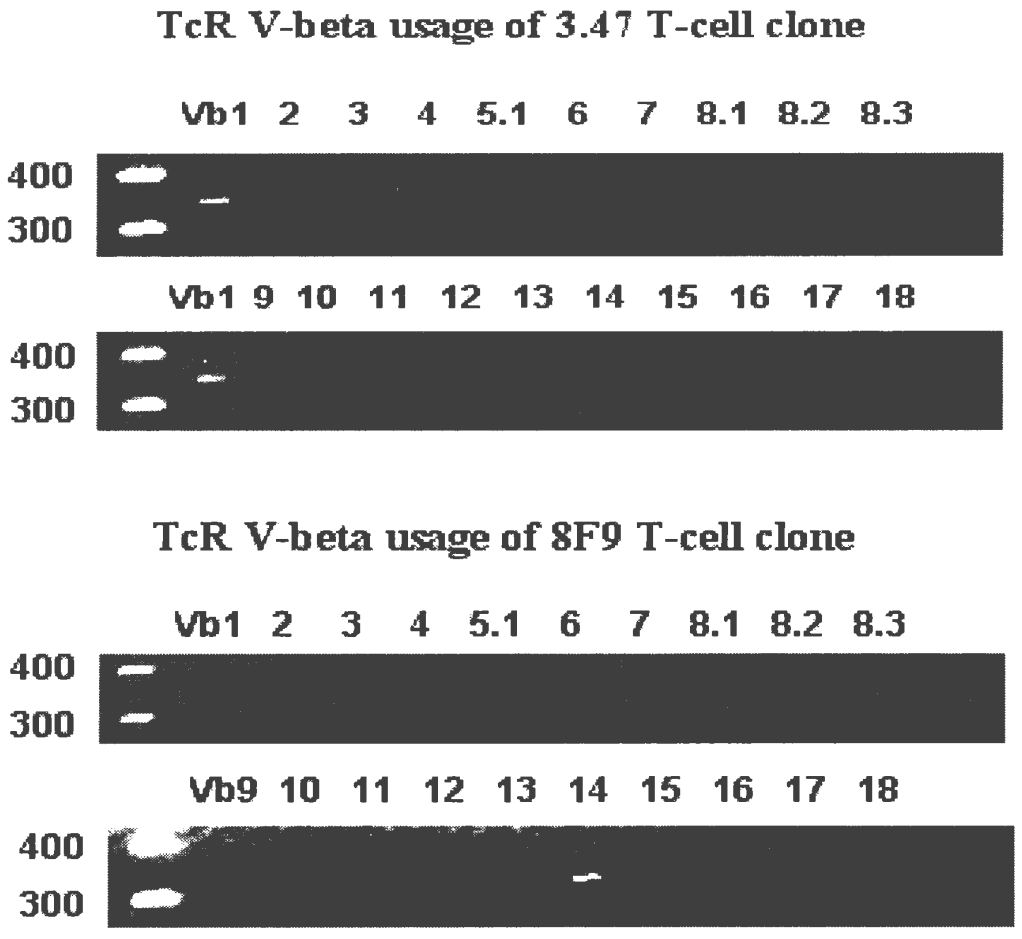

Fig. 4.4. The TcR Vβ family usage of 3.47 and 8F9 T-cell clones. Fresh mRNA isolated from 5 x 10^6 3.47 or 8F9 T-cells were used for 1st strand cDNA synthesis. PCR amplification was performed using the various Vβ family-specific forward primers and a Cβ2-specific reverse complementary primer. The PCR products were visualized in a 1.5% agarose gel.

Fig. 4.5. The VDJ region sequences of 3.47 and 8F9 TcR β-chains

Sequencing results for the VDJ region of the 3.47 TcR beta-chain

1	aagctacttt tacatatatc tgccgtggat ccagaagact cagctgtcta	*50*
51	tttttgtgcc agcagccatg atcgggacag ggactatgaa cagtacttcg	*100*
101	gtcccggcac caggctcacg gttttagagg atctgagaaa tgtgactcca	*150*
151	cccaaggtct ccttgtttga gccatcaaaa gcagagattg caaacaaaca	*200*
201	aaaggctacc ctcgtgtgct tggccagggg cttcttccct gaccacgtgg	*250*
251	agctgagctg gtgggtgaat gg	*272*

Sequencing results for the VDJ region of the 8F9 TcR beta-chain

1	ccactctggc ttctacctct gtgcctggag ttggcaagac acccagtact	*50*
51	ttgggccagg cactcggctc ctcgtgttag aggatctgag aaatgtgact	*100*
101	ccacccaagg tctccttgtt tgagccatca aaagcagaga ttgcaaacaa	*150*
151	acaaaaggct accctcgtgt gcttggccag gggcttcttc cctgaccacg	*200*
201	tggagctgag ctggtgggtg aatgg	*225*

Fig.4.6.A. The full TcR β-chain sequence of the 3.47 T-cell clone*

```
1      atgagctgca ggcttctcct gtatgtttcc ctatgtcttg tggaaacagc      50
51     actcatgaac actaaaatta ctcagtcacc aagatatcta atcctgggaa      100
101    gagcaaataa gtctttggaa tgtgagcaac atctgggaca taatgctatg      150
151    tactggtata aacagagcgc tgagaagccg ccagagctca tgtttctcta      200
201    caatcttaaa cagttgattc gaaatgagac ggtgcccagt cgttttatac      250
251    ctgaatgccc agacagctcc aagctacttt tacatatatc tgccgtggat      300
301    ccagaagact cagctgtcta tttttgtgcc agcagccatg atcgggacag      350
351    ggactatgaa cagtacttcg gtcccggcac caggctcacg gttttagagg      400
401    atctgagaaa tgtgactcca cccaaggtct ccttgtttga gccatcaaaa      450
451    gcagagattg caaacaaaca aaaggctacc ctcgtgtgct tggccagggg      500
501    cttcttccct gaccacgtgg agctgagctg gtgggtgaat ggcagggagg      550
551    tccacagtgg ggtcagcacg gaccctcagg cctacaagga gagcaattat      600
601    agctactgcc tgagcagccg cctgagggtc tctgctacct tctggcacaa      650
651    tcctcgaaac cacttccgct gccaagtgca gttccatggg ctttcagagg      700
701    aggacaagtg gccagagggc tcacccaaac ctgtcacaca gaacatcagt      750
751    gcagaggcct ggggccgagc agactgtgga atcacttcag catcctatca      800
801    tcagggggtt ctgtctgcaa ccatcctcta tgagatccta ctggggaagg      850
851    ccaccctata tgctgtgctg  gtcagtggcc tagtgctgat ggccatggtc      900
901    aagaaaaaaa attcctga                                        918
```

* Leader sequence: 1 - 54
V-region: 55 - 339
D-region: 340 - 354
J-region: 355 - 396
C-region: 397 - 918

Fig.4.6.B. The full TcR β-chain sequence of the 8F9 T-cell clone*

```
1     atgctgtact ctctccttgc ctttctcctg ggcatgttct tgggtgttag   50
51    tgctcagact atccatcaat ggccagttgc cgagatcaag gctgtgggca   100
101   gcccactgtc tctggggtgt accataaagg ggaaatcaag ccctaacctc   150
151   tactggtact ggcaggccac aggaggcacc ctccagcaac tcttctactc   200
201   tattactgtt ggccaggtag agtcggtggt gcaactgaac ctctcagctt   250
251   ccaggccgaa ggacgaccaa ttcatcctaa gcacggagaa gctgcttctc   300
301   agccactctg gcttctacct ctgtgcctgg agttggcaag acacccagta   350
351   ctttgggcca ggcactcggc tcctcgtgtt agaggatctg agaaatgtga   400
401   ctccacccaa ggtctccttg tttgagccat caaaagcaga gattgcaaac   450
451   aaacaaaagg ctaccctcgt gtgcttggcc aggggcttct tccctgacca   500
501   cgtggagctg agctggtggg tgaatggcag ggaggtccac agtggggtca   550
551   gcacggaccc tcaggcctac aaggagagca attatagcta ctgcctgagc   600
601   agccgcctga gggtctctgc taccttctgg cacaatcctc gaaaccactt   650
651   ccgctgccaa gtgcagttcc atgggctttc agaggaggac aagtggccag   700
701   agggctcacc caaacctgtc acacagaaca tcagtgcaga ggcctggggc   750
751   cgagcagact gtggaatcac ttcagcatcc tatcatcagg gggttctgtc   800
801   tgcaaccatc ctctatgaga tcctactggg gaaggccacc ctatatgctg   850
851   tgctggtcag tggcctagtg ctgatggcca tggtcaagaa aaaaaattcc   900
901   tga                                                     903
```

* Leader sequence: 1 - 45
V-region: 46 - 330
D-region: 331 - 336
J-region: 337 - 381
C-region: 382 - 903

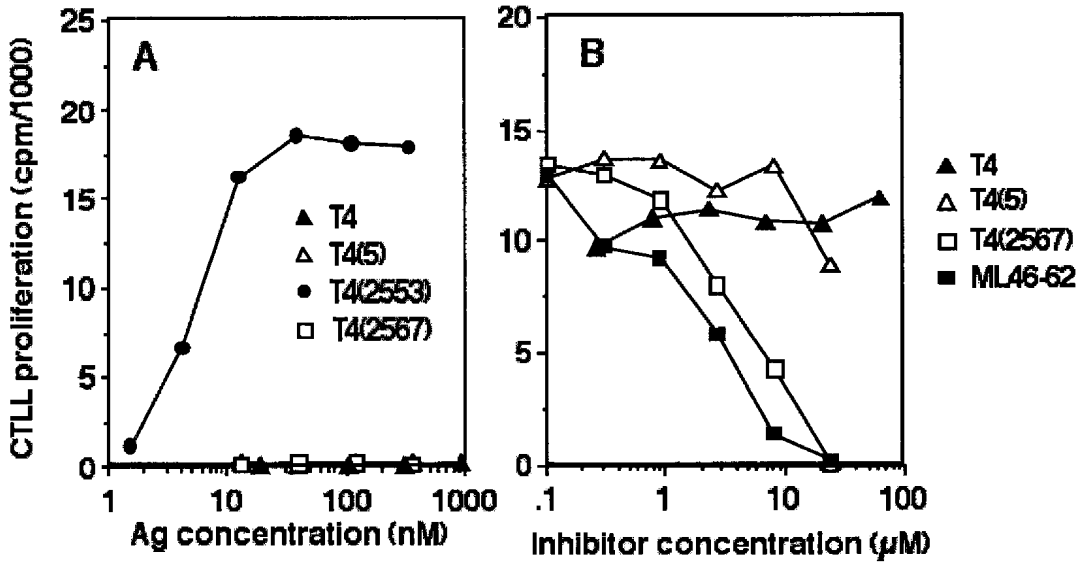

Figure 4.7. The presence of T4 within a Tg peptide is not sufficient to activate the 3.47 clone. *A,* The 3.47 clone does not recognize other T4-containing peptides of Tg. *B,* Competitive inhibition of 3.47 T-cell activation in the presence of 37.5 nM of T4(2553), TA3 APC and increasing amounts of inhibitor peptides. Control without inhibitory peptide is 14,830 cpm. Points indicate means of triplicate wells and denote the thymidine uptake (cpm) by the IL-2-dependent CTLL cells.

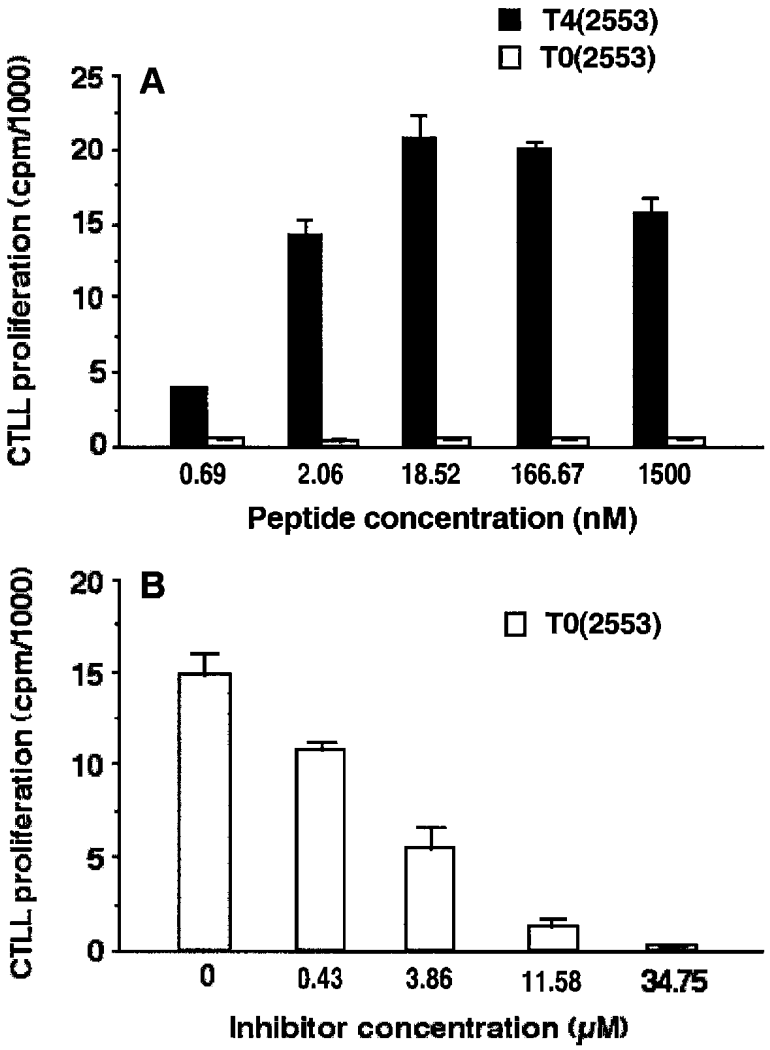

Figure 4.8. The 3.47 clone recognizes a Tg determinant modified by iodine atoms. *A,* Lack of 3.47 T-cell activation by the thyronine-containing analog peptide T0(2553); *B,* Competitive inhibition of 3.47 activation in the presence of 25 nM of T4(2553), TA3 APC and increasing amounts of the competitor analog peptide T0(2553). Data represent one of three separate experiments.

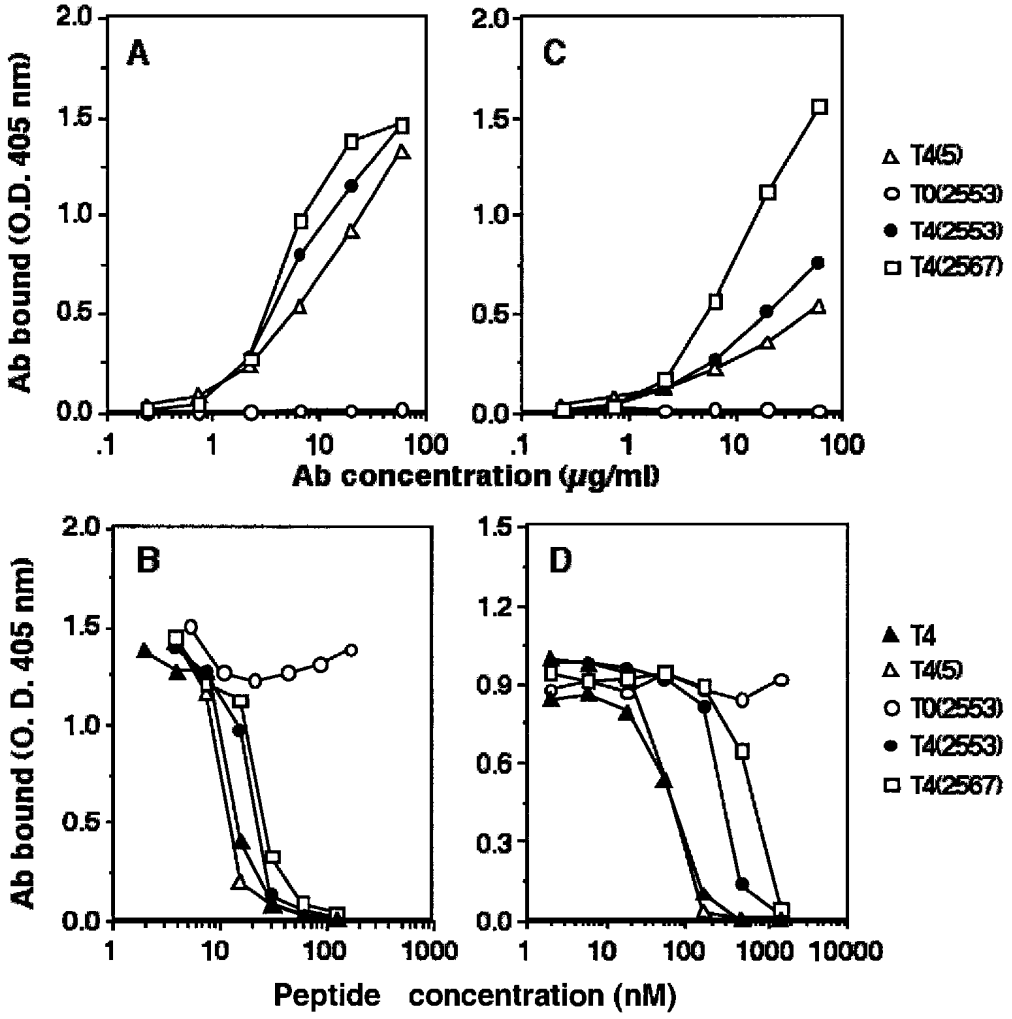

Figure 4.9. *A* and *C*, Reactivity of mAbs to Tg peptides. ELISA was performed using 1 µg peptide/well and serial dilution of 55H8 (*A*) and 91A1 (*C*) as shown. *B* and *D*, Competitive inhibition of mAbs binding to mouse Tg in the presence of T4 or Tg peptide. ELISA plates were coated with 1 µg Tg/well. Competitive inhibition was performed by using1.0 µg/ml of 55H8 (*B*) or 5.0 µg/ml of 91A1 (*D*) in the presence of serial dilution of peptides or T4 molecules. Points indicate means of triplicate wells and standard deviations are less than 10 % of the means.

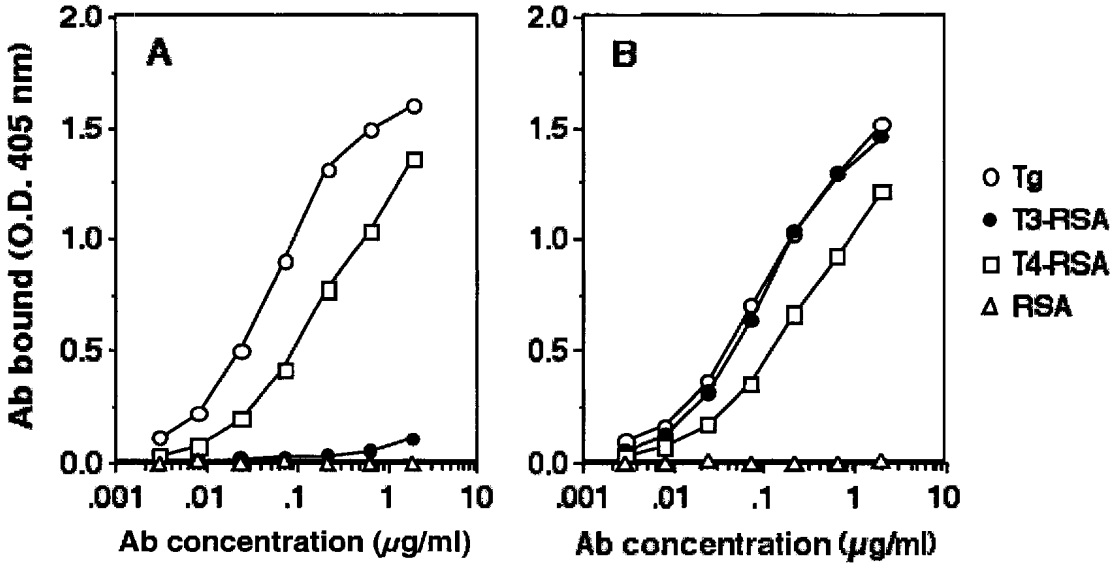

Figure 4.10. Fine specificity of the two T4-specific mAbs. Recognition of Tg and T4 or T3 carried by rabbit serum albumin (RSA) by mAb 55H8 (*A*) and 91A1 (*B*). ELISA was performed using 1 μg of Tg or RSA conjugates per well and serial dilution of mAbs as shown.

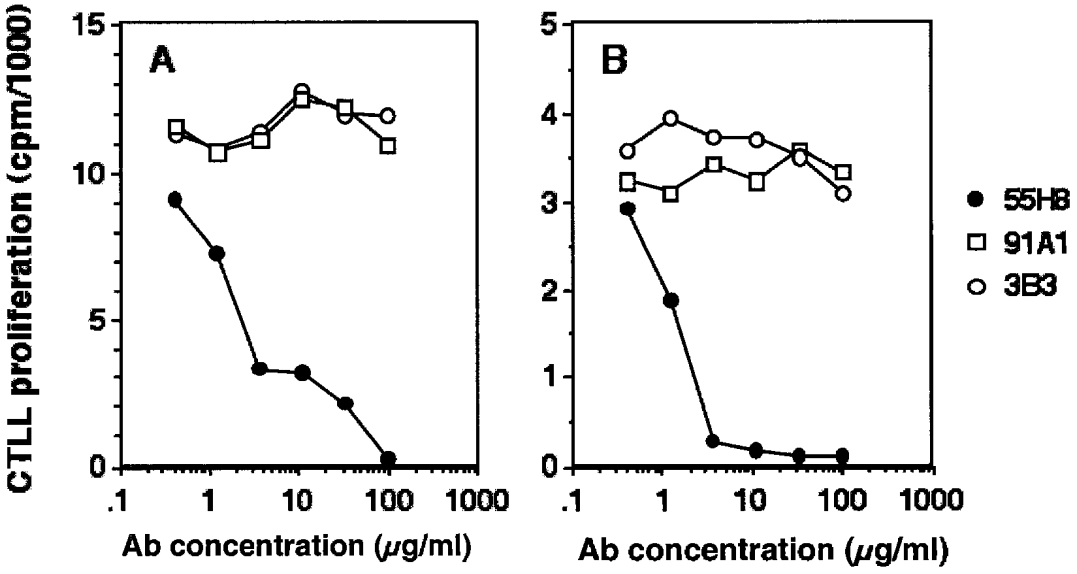

Figure 4.11. The T4-specific mAb 55H8 blocks recognition of the T4(2553) peptide by the 3.47 clone. The APC line TA3 (*A*) or thioglycollate-stimulated peritoneal macrophages from CBA mouse (*B*) were pulsed with T4(2553) (3 μg/ml) for 4 hr, washed and cultured with 3.47 cells in the presence of increasing amounts of the mAbs shown. Points indicate means of triplicate wells and reflect the relative amounts of IL-2 released in the culture supernatants following activation of the 3.47 cells. Data represent one of three separate experiments.

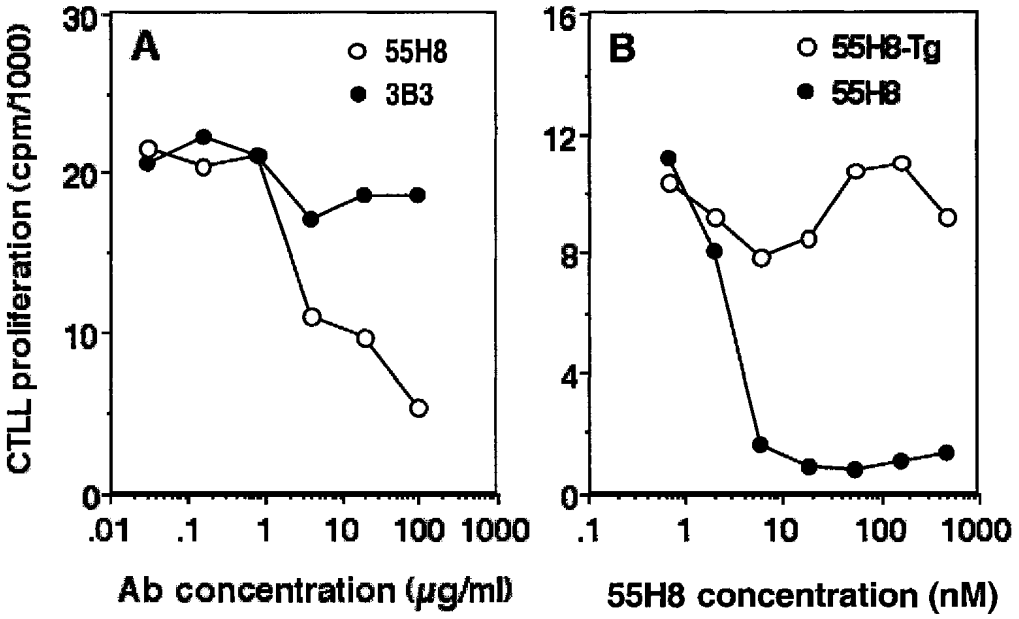

Fig.4.12. *A*. 55H8 blocks activation of 3.47 T-cells by the fixed, T4(2553)-pulsed APC.TA3 cells ($5x10^6$ cells / ml) were pulsed with 3 μg/ml of T4(2553) peptide for 2h followed with fixation by 0.05% glutaraldehyde for 30 seconds. The fixed, T4(2553)-pulsed TA3 cells ($5x10^5$ /well) were cultured with same number of 3.47 T-cells in the presence of serial dilution of blocking mAbs shown. CTLL proliferation assay was performed to compare IL-2-secretion by the 3.47 T-cells under different conditions. ***B***. The 55H8-Tg immune complex did not block the activation of 3.47 T-cells by T4(2553) peptide. The 55H8-Tg immune complex was preformed by mixing equimolar amount of 55H8 and Tg for 1 h before the assay. The concentration of IC was expressed as 55H8 concentration. To perform the activation assay, the TA3 cells ($5x10^6$/ml) were pulsed with 2 μg/ml of T4(2553) peptide for 6 h followed by washing. The pulsed TA3 cells were cultured with same number of 3.47 T-cells ($5x10^5$/well) in the presence of serial dilutions of either 55H8 mAb or the 55H8-Tg immune complex. The IL-2 content of the 24h-culture supernatants was assessed by CTLL proliferation. The control without blocking mAbs was 14,097 cpm.

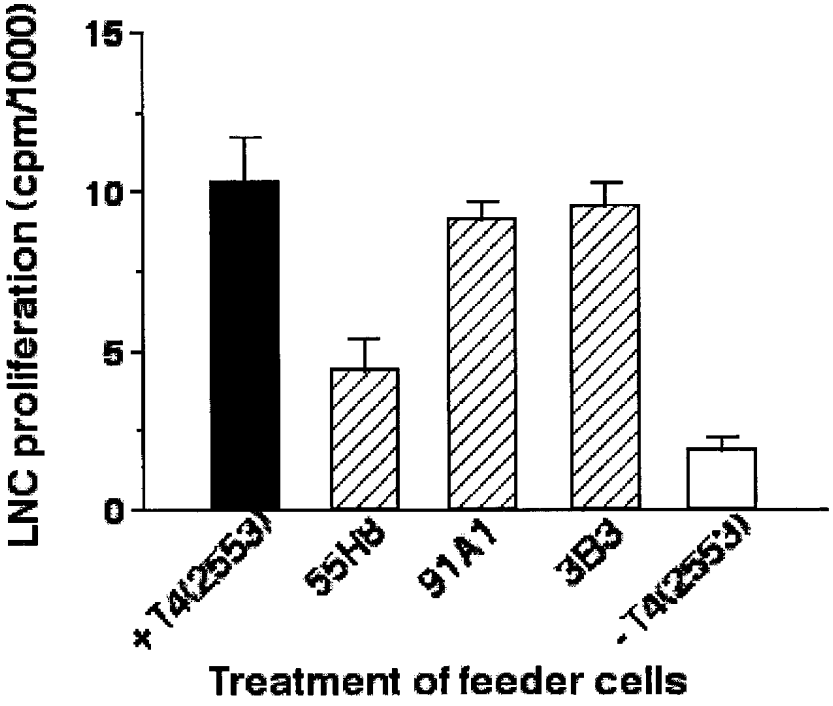

Figure 4.13. Blockade of activation of T4(2553)-primed LNC by T4(2553)-specific mAb 55H8. Mitomycin-treated normal CBA splenocytes were pulsed with or without 10 μg/ml of T4(2553) peptide for 6 hr. After washing, some of the T4(2553)-pulsed splenocytes were treated with 60 μg/ml of mAb 55H8, 91A1 or 3B3 for 1 hr, followed by complete washing. LNC from CBA mice immunized 9 days earlier with 100 μg T4(2553) peptide in CFA were cultured with T4(2553)-pulsed (Black bar), T4(2553)-pulsed and mAb-treated (Hatched bars), or non-pulsed (Empty bar) syngeneic splenocytes for proliferation assay as described in Materials and Methods.

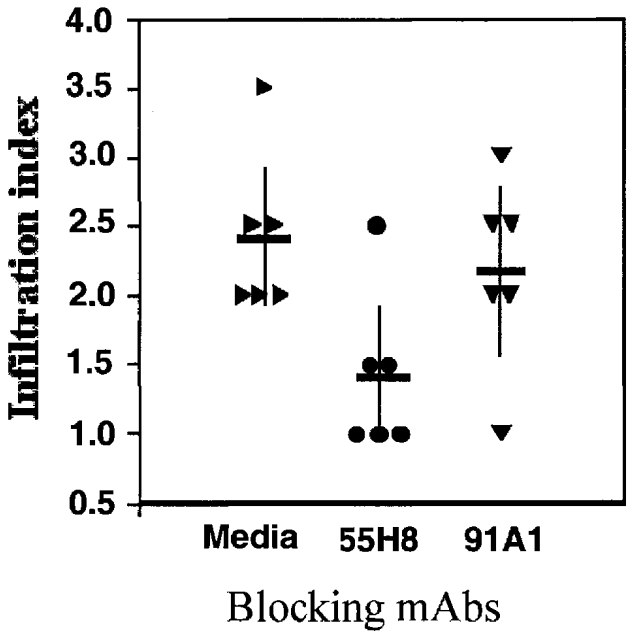

Fig. 4.14. 55H8 mAb ameliorates EAT transferred by T4(2553)-primed LNC. RBC-lysed and mitomycin-treated splenocytes ($2x10^7$ cells/ml) from CBA mice were pulsed with 30 μg/ml of T4(2553) peptide for 4h, followed with complete wash. The T4(2553)-pulsed splenocytes ($2x10^7$ cells/ml) were incubated with 100 μg/ml of mAb 55H8 or 91A1 or with media only for 2h (Variants are shown on the X-axis). After washing, the different groups of splenocytes ($2x10^7$ cells/ml) were mixed with T4(2553)-primed LNC ($4x10^7$ cells/ml) from CBA mice immunized with 100 μg of T4(2553) peptide emulsified in CFA for 10 days, and the mixture was cultured in 6-well plate in complete DMEM media for 4 days. Then, dead cells in the mixture were removed with Ficoll centrifugation, and living cells were collected. For adoptive transfer, 10^7 of living cells suspended in 1 ml of DMEM were injected i.p. into female CBA mice (6 mice per group). On day 13 after the transfer, the thyroid glands of the recipient mice were collected for scoring EAT severity. The lymphocyte infiltration indexes were scored blind as described in Materials and Methods. Horizontal bars represent the means of the infiltration indexes, and the vertical bars represent the standard deviations.

CHAPTER 5

ENHANCED IODINATION OF THYROGLOBULIN FACILITATES PROCESSING AND PRESENTATION OF A CRYPTIC PATHOGENIC PEPTIDE

(Submitted for publication)

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Yang Dai generated all the experimental data. Varada P. Rao generated T-cell hybridomas 5E8 and 6E10.

5.1. Abstract

Increased iodine intake has been associated with the development of experimental autoimmune thyroiditis (EAT), a T-cell mediated disease. The biological basis for this association remains poorly understood but an attractive postulate has been that enhanced incorporation of iodine in thyroglobulin (Tg), promotes generation of pathogenic T-cell determinants. Since most of the known pathogenic Tg T-cell epitopes are cryptic and do not contain iodine atoms, we sought in this study to directly test this hypothesis by using the pathogenic non dominant A^s-binding Tg peptides p2495 and p2694 as model antigens. SJL mice challenged with highly iodinated Tg (I-Tg) developed EAT of higher severity than Tg-challenged controls and LNC from I-Tg primed hosts showed a higher proliferation and IL-2 or IFN–γ release in response to I-Tg in vitro than Tg-primed LNC reacting to Tg. Interestingly, I-Tg-primed LNC proliferated strongly in vitro against p2495 but not p2694 indicating efficient and selective generation of p2495 following processing of I-Tg in vivo. Tg-primed LNC did not respond to either peptide. Similarly, the p2495-specific, IL-2 secreting T-cell hybridoma clone 5E8 was activated when cultured with I-Tg, but not Tg -pulsed splenocytes. Whereas, the p2694-specific T cell hybridoma clone 6E10 remained unresponsive to splenic APC pulsed with either Tg or I-Tg. The selective in vitro generation of p2495 was demonstrable in macrophages or dendritic cells but not B cells suggesting heterogeneity in the capacity of various APC sets to process I-Tg. These data demonstrate that enhanced iodination of Tg facilitates selective

processing and presentation of a cryptic pathogenic peptide in vivo or in vitro and suggest a mechanism that can account for the association of high iodine intake and development of EAT.

5.2. Introduction

Increased iodine (I) ingestion has been shown to promote induction of autoimmune thyroiditis in humans (Beierwaltes, 1969; Boyages et al., 1989; Braverman et al., 1971; Kahaly et al., 1998; Tajiri et al., 1986), and experimental animals such as chicken (Bagchi et al., 1985), rats (Allen et al., 1986; Mooij et al., 1993), hamsters (Follis, 1959) and mice (Rasooly et al., 1996). The mechanisms underlying this association remain poorly understood. One attractive theory has been that enhanced I intake increases the immunogenicity of thyroglobulin (Tg), precipitating an autoimmune response. This is a plausible explanation given that a) the main metabolic role of Tg is the organification of I and the formation of the hormones thyroxine (T4) and triiodothyronine (T3) via intramolecular coupling of specific iodotyrosyls (Dunn, 1995), b) Tg is the largest autoantigen known, inducing experimental autoimmune thyroiditis (EAT) in several species (Weetman and McGregor, 1994) and c) highly iodinated Tg (I-Tg) demonstrates enhanced immunogenicity (Sundick et al., 1987) or altered antigenicity (Saboori et al., 1998) at the serological level.

Tg-induced EAT, which serves as a model for Hashimoto's thyroiditis in humans, is nevertheless, considered to be a T-cell mediated disease (Weetman and McGregor, 1994) and the question whether or how incorporation of I into Tg promotes generation of Tg-specific autoreactive T cells has not been adequately investigated. Four out of the five known pathogenic Tg peptides do not contain I atoms or T4 residues (Carayanniotis and Kong, 2000; Carayanniotis and Rao, 1997). Furthermore, studies using peptide analogues that encompass hormonogenic - i.e. T4-containing sites of Tg have clearly shown that these epitopes are generated even after processing of normal Tg and that the presence of I atoms per se does not render a peptide immunogenic (Champion et al., 1991; Dawe et al., 1996; Kong et al., 1995). On the other hand, processing of non-iodinated Tg does not generate the pathogenic peptide (2549- 2560) in vitro (Champion et al., 1987), suggesting that a minimal amount of Tg iodination is required for the generation of some Tg T-cell epitopes.

In this study, we have examined whether transition of normal Tg to a highly iodinated form (I-Tg) – i.e. a shift from 15-20 I atoms to 55-70 I atoms per monomeric subunit - would enhance its immunopathogenicity in mouse EAT. The availability and prior characterization of two non dominant, A^s-binding, pathogenic Tg peptides (2495-2511) (p2495) and (2694-2711) (p2694) in our laboratory (Carayanniotis et al., 1994; Chronopoulou and Carayanniotis, 1992) has further allowed us to test the hypothesis that processing of I-Tg, but not Tg,

leads to generation of cryptic pathogenic determinants. Such a mechanism might partly account for the association of high I intake and autoimmune thyroiditis.

5.3. Results

5.3.1. Preparation of highly iodinated Tg (I-Tg)

The iodine content in the I-Tg preparations was assessed by a modified (Saboori et al., 1993), non-incinerative, colorimetric method (Palumbo et al., 1982) that is based on the catalytic function of iodine in the reduction of the yellow ceric ion Ce(IV) to the colorless cerious ion Ce(III) by arsenite As(III). Using thyroxine (T4) as a standard in the assay it was confirmed that the initial change in absorbance was proportional to the concentration of T4 over the range of 120 to 960 pmol **(Fig. 5.1A)**. By extrapolation, normal mouse Tg was shown to contain 15-20 I atoms and this content increased to a plateau of 70 I atoms per molecule over a 20 min incubation period with IODOGEN **(Fig. 5.1B).** Maximally iodinated Tg (55-75 I atoms per monomer) was used in all subsequent work.

5.3.2. Iodination enhances the immunogenicity and pathogenicity of Tg

To compare the immunogenicity of I-Tg vs. Tg, SJL/J mice were challenged s.c. with 100 μg Tg or I-Tg emulsified in CFA, and the antigen-specific in vitro

responses of primed LNC were determined 9-11 days later. It was observed that, upon in vitro stimulation with Tg, LNC from Tg-primed mice did not proliferate significantly (S.I. <3) and did not secrete detectable levels of IL-2 (< 52 pg/ml) or IFN-γ (< 366 pg/ml) in the culture supernatant (**Table 5.1**). In contrast, LNC from I-Tg-primed mice, proliferated strongly (S.I.= 12-15) against I-Tg in vitro, while secreting significant amounts of IL-2 (785-805 pg/ml) and IFN-γ (787-3654 pg/ml). Interestingly, I-Tg-primed LNC responded only weakly to Tg in vitro (**Table 5.1**) while no responses were detected against highly iodinated OVA (= 10 I atoms/molecule, data not shown), indicating recognition of determinants restricted mainly to I-Tg. These data suggested that modification of Tg by I rendered this molecule highly immunogenic at the T cell level.

To examine whether the increased imunogenicity of I-Tg correlates with its thyroiditogenicity, we immunized SJL/J mice (6 mice/group) with Tg or I-Tg in CFA on day 0, and boosted them with the respective Ag in IFA on day 14. Thyroids were collected on day 28 for histological examination of EAT development. Five out of six I-Tg-challenged mice developed severe EAT (infiltration index (I.I.)≥ 3) and the group exhibited a mean I.I.= 2.83 ± 0.98 (**Fig. 5.2**). In contrast, all Tg-challenged mice developed mild EAT (I.I. ≤ 2.0) and a significantly lower group I.I. mean (=1.33 ± 0.75, $p < 0.01$). These results demonstrated the increased pathogenicity of the I-Tg preparation.

5.3.3. Enhanced iodination of Tg promotes generation of the cryptic pathogenic peptide p2495

The enhanced immunopathogenicity of I-Tg vs Tg could be explained via at least two distinct mechanisms that are not mutually exclusive. First, iodination might facilitate generation of cryptic T-cell epitopes that are shared with normal Tg but are formed only after processing of I-Tg in APC. Second, iodination might lead to formation of new I-modified T-cell epitopes that do not exist in normal Tg. We proceeded to seek evidence for the first mechanism because of the availability and prior characterization of the cryptic Tg peptides (2495-2511) (p2495) and (2694-2711) (p2694) in our laboratory (Carayanniotis et al., 1994; Carayanniotis and Rao, 1997; Chronopoulou and Carayanniotis, 1992). These peptides are immunogenic and cause EAT but are not generated following processing of Tg in vivo or in vitro.

SJL/J mice were again immunized with Tg or I-Tg in CFA, and 9 days later, the draining LNC were tested in recall proliferative assays against p2495 or p2694 in vitro. Neither peptide could stimulate the Tg-primed LNC to proliferate, in accordance with their cryptic nature **(Fig. 5.3)**. Surprisingly, however, p2495 but not p2694 induced significant proliferation of I-Tg-primed LNC. This finding supported the view that iodination of Tg promotes selective generation of pathogenic peptides such as p2495. To confirm this at the clonal T-cell level we used the $CD4^+$, A^s-restricted 5E8 and 6E10 T-cell hybridoma clones that secrete

IL-2 upon recognition of p2495 and p2694, respectively **(Fig. 5.4A and B)**. Freshly isolated SJL spleen cells, used as APC, were incubated with 5E8 or 6E10 T cells and varying concentrations of Tg, I-Tg or I-OVA. It was observed that I-Tg activated strongly the p2495-specific 5E8 clone within the 0.1-1 μM range **(Fig. 5.4C)**, whereas equimolar amounts of normal Tg were not stimulatory. Similarly, I-OVA did not elicit a response up to 3.5 μM excluding the possibility that the high I content in the antigenic preparation mediated a non-specific activation of the T cells. On the other hand, the p2694-specific 6E10 clone was not activated with either Tg or I-Tg, **(Fig. 5.4D)** while it responded strongly to equimolar amounts of free p2694 (data not shown). These data confirmed that enhanced iodination of Tg promoted the selective processing and presentation of the cryptic pathogenic peptide p2495 in APC.

5.3.4. Generation of p2495 following processing of I-Tg in macrophages and DC

We next examined whether the selective generation of p2495 could be observed only after processing of I-Tg in certain APC subsets. Splenic resting B-cells were isolated by complement-mediated depletion of T-cells and accessory cells using anti-Thy1.1, anti-MAC-1 and anti-DC mAbs. Splenic macrophages were enriched by plastic adherence. Mature dendritic cells (DC) were obtained by culturing bone marrow progenitors in the presence of GM-CSF. The various APC populations were mixed at 1:1 ratio with 5E8 or 6E10 T cells in the presence of 1 μM Tg and I-Tg, using equimolar amounts of free p2495, p2694 and I-OVA as

controls. As shown in **Fig. 5.5**, none of the APC populations could generate p2495 or p2694 following processing of normal Tg, as indicated by the lack of activation of the peptide-specific T-cell clones. In contrast, processing of I-Tg in macrophages or dendritic cells but not B cells allowed the generation of p2495 and the activation of the 5E8 clone (**Fig. 5.5, A, C, E**). This was a selective process because p2694 was not generated in any APC under identical conditions of culture (**Fig, 5 B, D, F**). These findings demonstrated that enhanced iodination of Tg promotes selective generation of p2495 in specific APC subsets such as macrophages and DC which are potent stimulators of naïve T cells.

5.4. Discussion

Tg plays a central role in thyroid function and pathology because of its capacity a) to store available iodine, b) to act as a matrix for the formation and storage of thyroid hormones, and c) to encompass epitopes that activate infiltrating T cells that destroy the thyroid gland (Weetman and McGregor, 1994). In this study, we provide evidence that increased iodination of normal Tg renders the molecule highly immunogenic via at least one distinct mechanism: the enhanced generation of non-iodinated, cryptic, pathogenic T-cell determinants, such as p2495, in APC. Since the structure (Edelhoch et al., 1969), and proteolytic degradation (Fouchier et al., 1983; Lamas and Ingbar, 1978) of Tg are known to be affected by its iodine content, the present findings can be best explained by an

iodine-mediated modification of Tg substrate sites resulting in altered I-Tg processing in APC as compared to that of normal Tg. This hypothesis can account for the observed selective changes in the hierarchy (Sercarz et al., 1993) of T-cell epitopes in I-Tg, manifested by the generation of p2495 which changes from cryptic (Chronopoulou and Carayanniotis, 1992) to dominant and by the apparent lack of effect on the formation of p2694 which remains nondominant (Carayanniotis et al., 1994). The same hypothesis can also account for the differential capacity of various APC (macrophages and dendritic cells vs B cells) to process a given peptide, due to their diverse protease contents (Vidard et al., 1992). Because circulating Tg is poorly iodinated (Ikekubo et al., 1981; Schneider et al., 1983), peptides such as p2495 would not be normally generated in extrathyroidal sites, but once formed, they could elicit strong autoreactive T cell responses, as it is unlikely that immune mechanisms of peripheral tolerance would be pre-established against cryptic epitopes. This consideration deserves further merit in view of the fact that, so far, all known pathogenic T-cell epitopes in Tg have been characterized as nondominant (Carayanniotis and Rao, 1997). Our results are analogous to those of Griem et al. (Griem et al., 1996) who showed that alteration of bovine ribonuclease A by the metal ion Au(III) leads to sensitization of T cells specific for cryptic peptides.

Processing of I-Tg could also generate novel, iodine-modified T-cell determinants containing iodotyrosyls, or enhance the production of T-cell epitopes that encompass hormonogenic sites. While there is no evidence yet for the former

mechanism, the peptide (2549-2560) containing T4 at position 2553 (T4(2553) is known to activate pathogenic CD4+ T cells that recognize I atoms since they cannot be activated by a thyronine-containing (T0(2553)) analog that lacks iodine (Champion et al., 1991; Dawe et al., 1996; Hutchings et al., 1992; Kong et al., 1995). If there is increased production of T4(2553) during the processing of I-Tg vs Tg, it could potentiate generation of Tg-reactive, EAT-inducing T cells. However, the contribution of hormonogenic regions to the enhanced immunopathogenicity of I-Tg is likely to be minimal since most T4-containing peptides are weakly antigenic or devoid of immunogenicity (Carayanniotis and Kong, 2000).

High iodine intake may promote the iodination of Tg but it can also precipitate a number of other pathogenetic cascades related to iodine toxicity. High doses of iodine can cause necrosis of hyperplastic thyroid glands of normal animals (Bagchi et al., 1995; Follis, 1959) or human thyroid follicles in vitro (Many et al., 1992). This initial thyroid cell injury may be an important prerequisite for the subsequent development of autoimmune thyroiditis in goitrous NOD mice (Many et al., 1995), Obese Strain chickens (Bagchi et al., 1995) or humans with pre-existing goiters (Kahaly et al., 1998; Mizukami et al., 1993; Tajiri et al., 1986). In addition, iodine can exhibit pleiotropic effects by influencing processes that interfere with immune function. These include inhibition of IFN-γ-induced class II expression on rat (FRLT-5), but not human, thyroid cells (Davies et al., 1989), increase in the inducibility of the 72-kDa heat shock protein in cultured human

thyrocytes (Sztankay et al., 1994) and enhancement of IgG production in PWM-stimulated human peripheral blood lymphocytes in vitro (Weetman et al., 1983).

Our findings are in agreement with studies reporting an essential role of iodine for Tg recognition by human (Rasooly et al., 1998) T cells. Presentation of cryptic I-Tg peptides on professional APC such as dendritic cells would lead to activation of resting autoreactive T-cells that can subsequently act as effector cells in autoimmune thyroiditis and/or as T helper cells in the concomitant antibody response against thyroid antigens. In conjunction with the available TcR repertoire, the host MHC haplotype would dictate, via determinant selection, the relative contribution of each peptide in the disease process. This hypothesis would explain why only certain individuals develop autoimmune thyroiditis after high iodine intake (Weetman and McGregor, 1994) or why they occasionally present with enhanced B-cell autoreactivity to Tg without overt symptoms of thyroiditis (Boukis et al., 1983). The validity of this hypothesis will be further tested following the discovery of additional pathogenic T-cell epitopes in Tg.

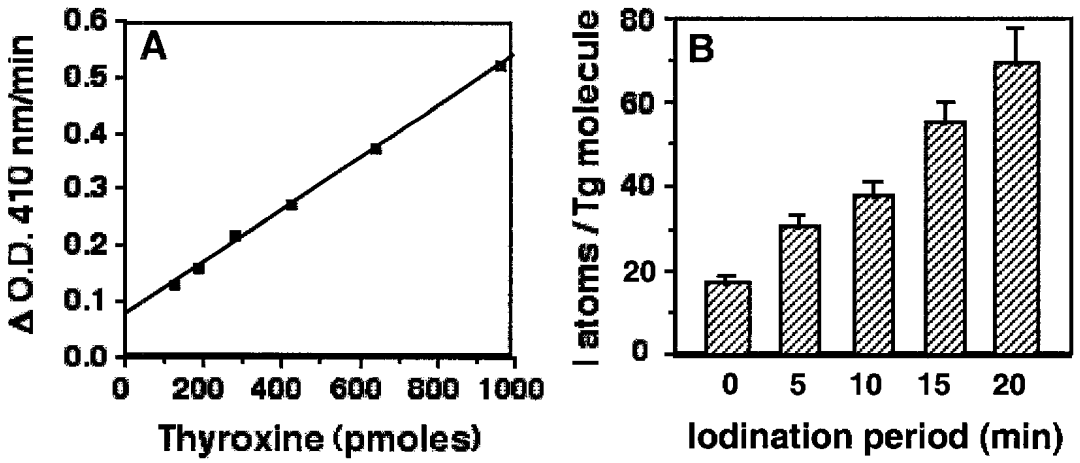

Fig. 5.1. Iodine determination for Tg and I-Tgs. *A,* T4 standard curve. The reduction of absorbance at 410 nm in the first 60 seconds of iodine-catalyzed Ceric-Arsenite reaction is shown for various amounts of T4 added in the reaction. *B,* Iodination increases the iodine contents in Tg. Tg was iodinated by IODOGEN method for various periods as shown, and 16 μg of the Tg or I-Tg samples was used for iodine determination. Iodine content of Tg was extrapolated from A (one mole of T4 equals 4 moles of iodine).

Table 5.1. Iodination of Tg enhances its immunogenicity

Exp. No.	Ag in vivo	Ags in vitro					
		Tg			I-Tg		
		SI	IL-2*	IFN-γ*	SI	IL-2	IFN-γ
Exp.1	Tg	2.9±0.3	52±8.8	<278	5.7±0.7	119±5.3	<278
	I-Tg	5.8±1.5	133±3.5	325±47	14.9±1.7	785±14	786.9±38
Exp.2	Tg	2.1±0.4	46±9	<366	3.3±0.2	87±8	<366
	I-Tg	2.9±0.6	131±15	<366	12.4±0.5	805±23	3654±62

* IL-2 & IFN-γ: pg/ml

Background cpm (cultures with no Ag) were: Tg-primed LNCs = 614 (740), I-Tg-primed LNCs = 1208 (1355); Detection limit for IL-2 was 52 (46) pg/ml; IFN-gamma was 278 (366) pg/ml. [*Exp.1* (*Exp.2*)]. IL-4 in all culture supernatants was undetectable.

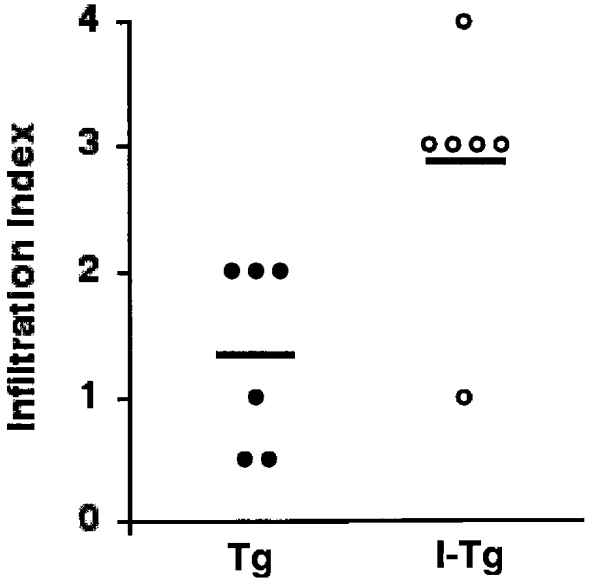

Fig. 5.2. Direct induction of EAT by immunization with Tg or I-Tg. Six female 6-8wk SJL mice per group were immunized with 100 μg of either Tg or I-Tg emulsified in CFA. After 2wk, the mice were boosted with 50 μg of the same Ags emulsified in IFA. The thyroid glands were collected at 4wk for histology examination. Scoring the slides for lymphocyte infiltration indexes was performed blind as described in Materials and Methods. ($p < 0.01$)

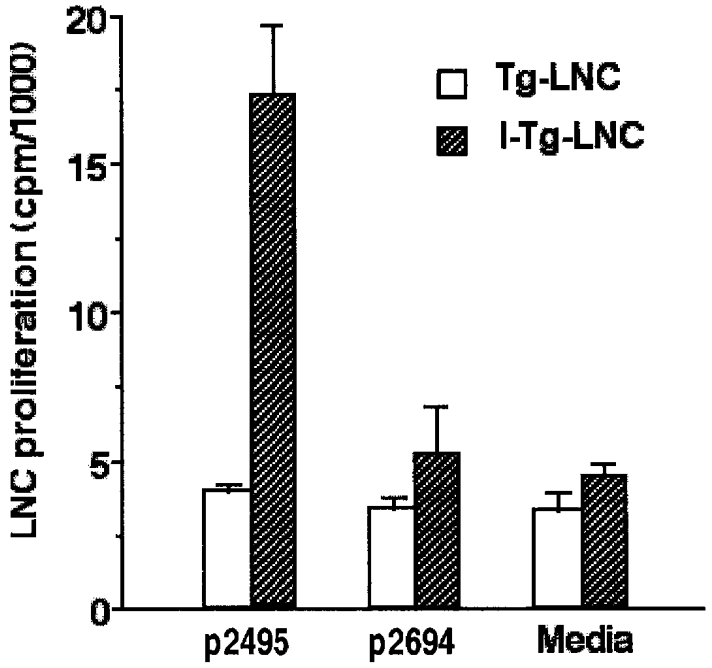

Fig. 5.3. Generation of the cryptic peptide, p2495, following immunization with I-Tg. Two female SJL mice per group were immunized with 100 μg of either Tg or I-Tg emulsified in CFA. After 10 days, the draining LNC ($5x10^5$ cells/well) were cultured with of each of Tg peptides (20 μg/ml) or media in total 200 μl/well of complete media. Proliferation of the LNC was expressed as the ^{3}H-thymidine incorporation in last 24 h of total 96 h culture. Each value represents average of triplicate wells.

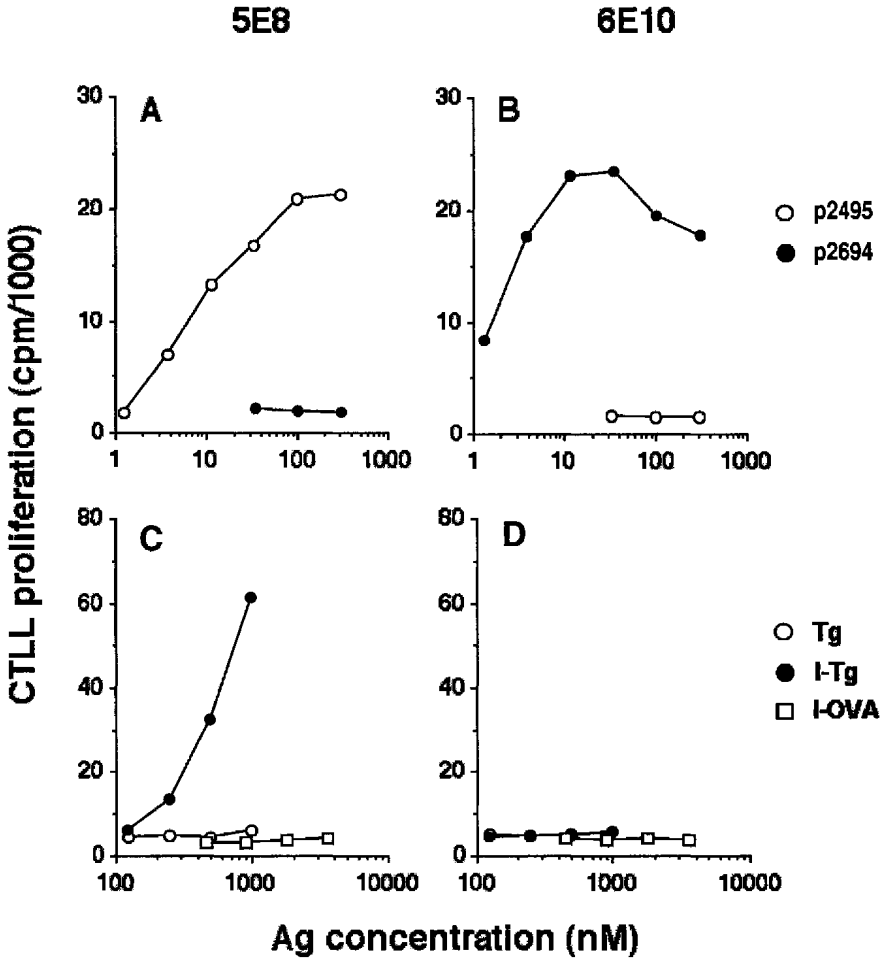

Fig. 5.4. Enhanced presentation of p2495 following processing of I-Tg. *A&B,* Specificity of Tg peptide-reactive T-cell hybridomas. H-2^s-restricted T-cell hybridomas 5E8 (A&C, p2495-specific) and 6E10 (B&D, p2694-specific) cells (10^5 /well) were cultured in 1:5 ratio with syngeneic splenocytes in the presence of serial dilutions of Ags as shown. IL-2-release from the T-cells was determined by CTLL proliferation assay.

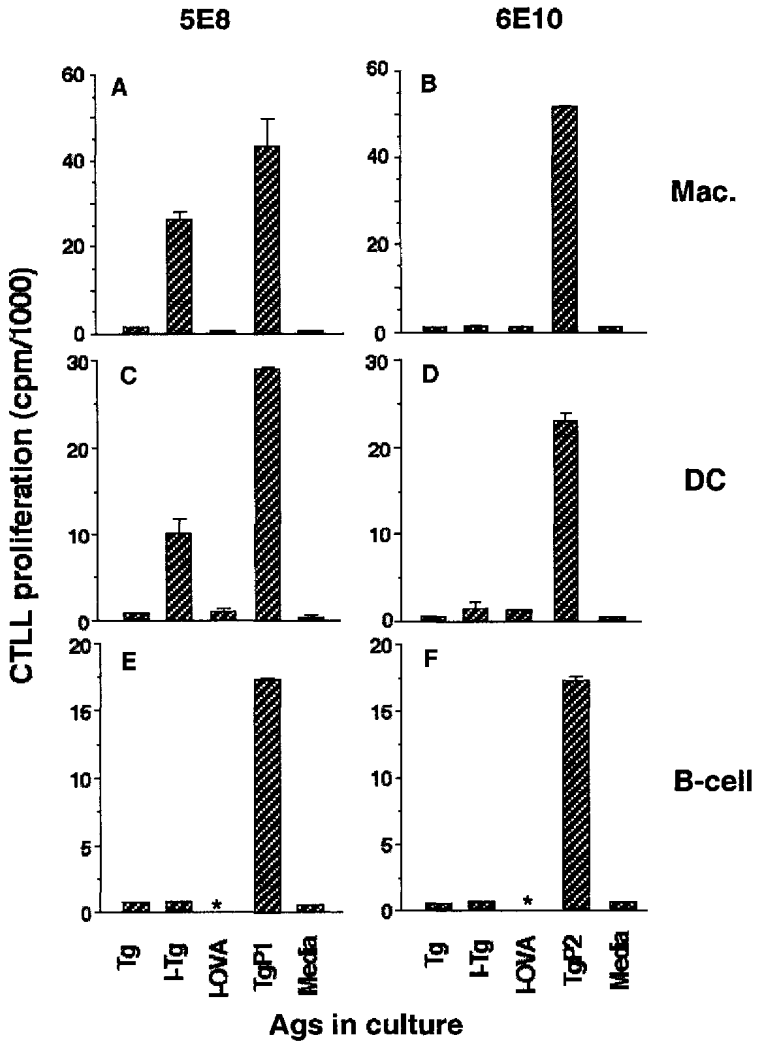

Fig. 5.5. Macrophages and DC are efficient in generation of p2495 following processing of I-Tg. Splenic macrophages, B-cells and bone-marrow-derived DC were isolated from SJL/J mice as described in Materials and Methods. Activation of T-cell hybridomas 5E8 (A, C and E, p2495-specific) and 6E10 (B, D and F, p2694-specific) was performed by culturing the hybridoma cells (10^5 cells/well) at a 1:1 ratio with the various APC in the presence or absence of 1 μM Ag as shown (*: not done). IL-2-release from the T-cells in the 24h-culture supernatants was assessed by CTLL proliferation assay. Each value represents average of triplicate wells, and figures shown are one of 3 experiments with similar results.

CHAPTER 6

FUTURE DIRECTIONS

The present study was initiated following the observation that none of the three pathogenic T-cell epitopes in Tg, Tg(2495-2511), Tg(2694-2711) and T4(2553), were immunodominant. All three peptides can directly induce EAT in mice following immunization with the peptides emulsified in CFA, but LNC primed with these peptides cannot recognize intact Tg in *in vitro* recall assays. The question raised at the beginning of the study was whether alterations of Tg processing in APC could facilitate generation of these cryptic or non-dominant epitopes. The data obtained demonstrate that processing of Tg-mAb ICs or iodinated Tg by certain APC populations can generate some of these non-dominant Tg epitopes. The study provides insights into mechanisms, such as post-translational modification of auto-Ag and epitope spreading, operating in T-cell-mediated organ-specific autoimmune diseases. This work has also raised new questions.

6.1. Regulation of T-cell response to Tg peptides using Tg-specific mAbs

Auto-Abs against Tg are commonly present in HT patients (Nordyke et al., 1993). The results in Chapter 3 clearly demonstrate that Tg-specific Abs can influence the processing and presentation of T-cell epitopes of Tg in vitro, suggesting that they may have an important immunoregulatory role during the course of HT. Tg-specific B-cells may play a central role in T-cell epitope spreading, a phenomenon commonly observed in T-cell-mediated autoimmune diseases such as EAE (Lehmann et al., 1992; Mamula et al., 1994). Tg-specific B-cells carry sIgs that recognize various binding sites on Tg, and following Tg internalization and processing, different B-cell clones may present different epitopes to Tg-specific T-cells through a mechanism proposed in our study. Therefore, the T-cell response against Tg can be diversified due to the activation of different Tg-specific B-cells. One objective in future studies will be to examine whether Tg-specific B-cells can selectively present T-cell epitopes of Tg and regulate the development of EAT.

6.1.1. Generation of T4(2553) peptide following processing of Tg by Tg-specific B-cells

Hypothesis: Tg-specific B-cells can efficiently internalize Tg and generate T4(2553) peptide.

Rationale: LNC primed in vivo with the T4(2553) peptide cannot proliferate in vitro in the presence of Tg but can be activated strongly by the priming peptide in culture (Champion et al., 1991). These data suggest that generation of T4(2553) peptide is not efficient following processing of Tg by APC in vitro. However, immunization of CBA/J mice with Tg emulsified in CFA can activate T-cells reactive to the T4(2553) peptide (Hutchings et al., 1992; Kong et al., 1995). Processing of Tg in vivo may generate sufficient T4(2553) to activate peptide-specific T-cells. Based on our observations in Chapter 3 that Tg-specific mAbs can enhance generation of T4(2553) following processing of Tg-Ab ICs in APC (Dai et al., 1999), we propose that Tg-primed B-cells are efficient in processing Tg to generate T4(2553) peptide.

Experimental design 1: To examine the efficiency of Ag processing and presentation in Tg-primed B-cells, the T-cell hybridoma 3.47 specific for T4(2553) will be used to monitor the generation of T4(2553). Tg-primed B-cells will be collected from the spleen of CBA/J mice previously challenged with Tg and LPS. B-cells will be purified by positive selection with anti-B220-coated magnetic beads. A control Ag such as OVA will be used to generate OVA-specific B-cells. Tg internalization in B-cells will be monitored directly using ^{125}I-labeled Tg. The activation assay will be performed by co-culturing 3.47 T-cells with Tg or OVA –specific B-cells in the presence of Tg.

Interpretation of results: If B-cells primed only with Tg but not OVA can activate the 3.47 T-cells in the presence of Tg in culture, we will conclude that Tg-primed

polyclonal B-cells can selectively enhance generation of T4(2553) during processing of Tg, perhaps due to their capacity to internalize large quantities of Tg. This finding will also support the hypothesis that Tg-primed B-cells may generate T4(2553) in vivo.

Experimental design 2: The hypothesis can be further addressed by examining whether Tg-primed B-cells have the capacity to activate T4(2553)-specific effector T-cells. T-cells collected from the draining LN of CBA/J mice immunized with T4(2553) will be used as responder cells. T-cells will be isolated by positive selection with anti-Thy-1-coated magnetic beads. Tg- or OVA-specific B-cells can be obtained from Ag-primed splenocytes following purification with anti-B220-coated magnetic beads. Activation of T4(2553)-primed T-cells will be done by culturing the enriched T4(2553)-primed T-cells with B-cells primed with Tg or OVA in the presence of Tg in culture. Generation of T4(2553)-primed effector T-cells will be demonstrated by adoptive transfer of the cultured T-cells into syngeneic naïve recipient mice.

Interpretation of results: Development of EAT in mice receiving T4(2553)-primed T-cells will confirm the generation of pathogenic effector T-cells in culture. If mice receiving T4(2553)-primed T-cells cultured with Tg and Tg-primed B-cells but not OVA-primed B-cells develop EAT, it will further support that generation of T4(2553) by Tg-primed B-cells are sufficient to activate T4(2553)-specific effector T-cells.

6.1.2. In vivo effects of 5D2 and 3C4 mAbs on activation of T-cells specific for T4(2553)

Hypothesis: 5D2 and 3C4 mAbs have an immunofocusing effect on activation of T4(2553)-reactive T-cells in vivo.

Rationale: In Chapter 3, it was shown that processing of 5D2-Tg and 3C4-Tg ICs in the B-cell line, TA3, enhances the generation of T4(2553). Whether this in vitro phenomenon represents a physiological mechanism, i.e. it can be mediated by normal B-cells, remains unkown.

Experiment design 1: To examine whether normal B-cells can process Tg-5D2 and Tg-3C4 ICs efficiently to generate T4(2553) peptide, resting B-cells from the spleens of normal CBA/J mice will be used as APC. Purification will be performed as above using anti-B220 coated magnetic beads. Tg-Ab ICs will be formed as described in Chapter 2. ICs, formed with the Tg-specific mAb 3B3 that is unable to enhance generation of T4(2553) shown in Chapter 3, will be used as controls. The rate of internalization of Tg-Ab ICs by normal B-cells will be directly measured by pulsing the B-cells with ICs of ^{125}I-labelled Tg with mAbs. The 3.47 T-cell hybridoma will be used to monitor the presentation of T4(2553) following processing of Tg or Tg-Ab ICs, and activation of the T-cells will be examined by measuring the IL-2 concentration in the culture supernatant.

Interpretation of results: If B-cells cultured with Tg-5D2 and Tg-3C4 but not Tg-3B3 activate the 3.47 T-cells, supportive evidence will be provided that normal B-cells

may process Tg-5D2 and Tg-3C4 ICs efficiently and amplify the T4(2553)-specific T-cell response.

Experiment design 2: Tg-Ab ICs will be used for immunization and induction of EAT in CBA/J mice. Tg-3B3 ICs will be again used as a control. On day 9 to 12 after immunization, draining LN will be collected for proliferation assay against T4(2553) in vitro. For induction of EAT, CBA/J mice will be s.c. immunized with Tg-Ab ICs emulsified in CFA, and boosted with same Ag in IFA after 2 weeks. Development of EAT will be examined by scoring the lymphocytic infiltration of thyroid glands in the second week after the boosting.

Interpretation of results: If LNC primed with Tg-5D2 and Tg-3C4 proliferate more strongly than Tg-3B3 –primed LNC against T4(2553) in vitro, this will support the view for an enhancing effect of 5D2 and 3C4 on generation of T4(2553) peptide following processing of Tg-Ab ICs in vivo. Increased EAT severity in mice immunized with Tg-5D2 and Tg-3C4, vs Tg-3B3-primed mice, will further confirm the immunofocusing effect of 5D2 and 3C4 on activation of T4(2553)-reactive T-cells.

6.1.3. Tg processing in B-cells with same specificity as 5D2 and 3C4 mAbs

Hypothesis: B-cells carrying sIg of the same specificity as 5D2 and 3C4 mAbs are efficient in processing of Tg and generation of T4(2553).

Rationale: It was shown in Chapter 3 that the B-cell hybridoma TA3 cannot process Tg to generate T4(2553). However, this peptide was generated efficiently in the same APC following processing of Tg complexed to 5D2 and 3C4 but not 3B3 mAbs. This enhanced presentation of T4(2553) requires FcR-mediated Ag internalization. It is not clear whether Tg internalized through BcR will be processed similarly as that through FcR. In other words, can different Tg-specific B-cell clones selectively generate different T-cell epitopes during Tg processing?

Experimental design: To construct B-cells with the same specificity as 5D2, 3C4 and 3B3 mAbs, the variable regions of the H- and L- chains of each mAb will be cloned from the respective mAb-secreting B-cell hybridoma. Then, the variable region cDNA will be subcloned into vectors containing the H- or L- chain constant regions of sIg to form chimeric Ig genes (Goodnow et al., 1988). TA3 B-cell hybridoma will be transfected with each of the chimeric sIg cDNA. Stable TA3 transfectants expressing the chimeric sIg of 5D2, 3C4 and 3B3 will be selected for Ag processing. To examine whether these transfected B-cell clones carrying the different chimeric sIgs can selective present T4(2553) peptide following processing of Tg, activation assay will be performed by co-culturing the 3.47 T-cells with the B-cell clones in the presence of Tg. Since the sIg expression level on the transfected B-cells may affect the rate of Tg internalization and, subsequently, the generation of T4(2553), expression of sIg on these B-cell clones will be examined using fluorescence-labeled anti-IgG. B-cell clones expressing similar levels of sIg will be used as APC. Tg binding and internalization in these B-cells will be directly measured with ^{125}I-labeled Tg.

Interpretation of results: If the B-cells of the same specificity as 5D2 and 3C4 but not 3B3 activate the 3.47 T-cells in the presence of Tg, we will obtain evidence that diverse Tg-specific B-cell clones may vary in their capacity to generate T4(2553). That is, generation of T4(2553) will not be simply a result of enhanced Tg uptake by Tg-specific B-cells.

6.2. Regulation of autoreactive T-cells: using TcR transgenic mice expressing a TcR specific for T4(2553)

EAT is T-cell-mediated autoimmune disease, and it is still unclear how auto-reactive T-cells escape from thymic selection and become activated in periphery. Since autoreactive T-cells causing autoimmune diseases are commonly polyclonal and recognize unknown epitopes, it is difficult to examine their development and fate during the onset of the disease. TcR transgenic mice provide a unique tool to overcome this difficulty. In these mice, most of circulating T-cells express a single TcR reactive with a known epitope of a self-Ag. TcR transgenic mice have been used with success in several autoimmune disease models, such as IDDM and EAE (Goverman et al., 1993; Katz et al., 1993), and have aided in the elucidation of mechanisms of T-cell maturation and regulation of autoimmune responses. However, so far, TcR-transgenic mice have not been established in EAT. The T-cell hybridoma 3.47 that has been characterized in Chapter 4 is an excellent material for the construction of a TcR-

transgenic mouse model, since it carries a TcR specific only for the T4-containing Tg peptide T4(2553) but not for the tyrosine-containing analog (Dawe et al., 1996). Such a TcR transgenic mouse model will be extremely valuable in EAT studies, concerning the maintenance of peripheral T-cell tolerance and recognition of post-translationally modified T-cell antigens.

6.2.1. Generation of transgenic mice expressing the TcR of the 3.47 clone

The TcR β-chain of the 3.47 clone consists of Vβ1, Jβ2.6. The VDJ sequence was shown in **Fig. 4.5**. TcR Vα and Jα usage by the 3.47 clone will be identified using the "anchored PcR" method.

Construction of α- and β- chain vectors:

Forward and reverse primers flanked with restriction sites will be designed to amplify the complete VJ region of α-chain or VDJ region of β-chain from the 3.47 genomic DNA sample. The PCR fragments will be ligated into pT_{α}cass and pT_{β}cass vectors that direct expression of TcR α-chain and β-chain genes, respectively, in transgenic mice (Kouskoff et al., 1995). Several clones will be sequenced to ensure that proper ligation of VJ or VDJ sequence into the vectors has been achieved. The functionality of the α- and β-chain constructs will be examined by transfection of the pT_{α}cass and pT_{β}cass vectors containing 3.47 α- and β-chain genes into the BW5147 $\alpha^{-}\beta^{-}$ thymoma or into a T-cell hybridoma with different specificity from 3.47 such as 8F9. Activation of the TcR-transfectants by

T4(2553) in the presence of A^k-expressing APC such as TA3 will provide evidence for the functionality of 3.47 α- and β- chain vectors.

Generation of founder mice:

Linearized TcR dsDNA from both the $pT_\alpha cass$ and $pT_\beta cass$ vectors will be co-injected into fertilized B6 oocytes to generate TcR transgenic founders with the EAT resistant B6 background as described by Kuchroo's group (Waldner et al., 2000). The TcR transgenes will be introduced into the EAT-susceptible strain CBA/J mice by backcrossing for at least 5 generations. Genotyping of the TcR transgenes will be done by Southern analysis using probes specific for the VJ of α-chain and the VDJ of β-chain genes of 3.47 clones. DNA from 3.47 and 8F9 T-cell clones will be used as positive and negative controls, respectively. The phenotype of the TcR-transgenic mice will be examined by proliferation assay of the peripheral lymphocytes against T4(2553) peptide.

6.2.2. The role of iodine in the development of spontaneous autoimmune thyroiditis (SAT) in the TcR transgenic mice

Hypothesis: Development of SAT in the T4(2553)-TcR transgenic CBA/J mice is iodine-dependent.

Rationale: It has been well-documented that post-translational modification of auto-Ags can increase their immunogenicity leading to autoimmune disease (Mamula et al., 1999; Molberg et al., 1998). Iodination of Tg molecules is an important post-translational modification that takes place in thyrocytes. Excess iodine intake has long been considered as a causative environmental factor of HT

(Rose et al., 1997). Roitt et al. proposed that Tg iodination facilitates the generation of new iodine-containing T-cell epitopes. We plan to examine the role of iodine in SAT development in TcR-transgenic mice enriched with T4(2553)-specific T-cells. This study will generate valuable data for understanding the role of iodine on development of HT.

Experiment design 1: The anti-Tg titer in the sera of the T4(2553)-TcR transgenic CBA/J mice will be tested by ELISA at different ages of the mice raised on normal diet. Mice of different ages will be sacrificed for histological examination of thyroid lymphocytic infiltration. The 5^{th} generation of the backcrossing littermates expressing either TcR α-chain or TcR β-chain single transgene will be used as control for development of SAT. The incidence and severity of SAT will be used as indexes for comparing the development of SAT between the TcR transgenic mice and their normal littermates.

Interpretation of results: If T4(2553)-TcR transgenic mice develop SAT early in their life when they are raised on normal diet, then this result will support the view that generation of T4(2553) peptide following Tg processing in vivo is sufficient to activate the transgenic T-cells.

Experiment design 2: To examine whether iodine plays a role in the development of SAT in the TcR-transgenic mice, mice will be divided into 3 groups as follows: Mice in Group 1 will be fed with drinking water containing TPO inhibitor 3-amino-1,2,4-triazole (ATA), which blocks iodination of Tg and hormonogenesis

in thyroid glands; Mice in group 2 will be treated with normal diet and water; Mice in Group 3 will be treated with iodine-enriched diet by adding 0.05% iodide in the drinking water. Development of SAT will be assessed by the serum Tg-specific IgG level and thyroid lymphocyte infiltration. Initiation of treatment and examination of SAT may differ depending on the age and severity of SAT in mice raised on normal diet.

Interpretation of results: Mice treated with ATA cannot synthesize T4 at position 2553. Therefore, decreased iodine uptake will prevent the development of SAT in the TcR-transgenic mice. If excess iodine intake exacerbates the development of SAT in the TcR-transgenic mice, then this result will support the concept that increased iodination of Tg can facilitate the generation of T4(2553) either by enhanced presentation of the peptide during Tg processing or by generation of more T4(2553)-containing Tg during hormonogenesis in thyrocytes.

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