STUDIES ON THE DEVELOPMENT OF A MOUSE MODEL OF GRAVES' DISEASE

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Studies on the development of a mouse model of Graves' disease

by

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ABSTRACT

Thyroid diseases are commonly caused by an autoimmune response to thyroid antigens. Hashimoto's thyroiditis (HT) is a T-cell mediated disease that ensues when the thyroid gland is destroyed by infiltrating lymphocytes specific antigens such as thyroglobulin (Tg) or thyroid peroxidase (TPO), leading to hypothyroidism. In Graves' disease (GD), autoantibodies against the thyroid-stimulating hormone receptor (TSHR) mimic the action of thyroid-stimulating hormone (TSH) and stimulate thyrocytes to overproduce thyroid hormones resulting in hyperthyroidism. Both spontaneous and experimentally induced animal models of HT exist and have contributed considerably to our understanding of the disease process. In contrast, difficulties in inducing TSHR-specific, stimulating antibodies have delayed the establishment of an animal model for GD. Recently, new strategies including immunization with TSHR-transfected fibroblasts and genetic immunization with eukaryotic expression vectors containing TSHR cDNA have provided promising results in mice, but further studies are required to optimize disease induction.

The research described herein focuses on establishing a mouse model of GD through intradermal DNA immunization of BALB/cJ and AKR/J mice with varying doses of plasmid vectors carrying the homologous (murine) or heterologous (human) TSHR cDNA (Chapter 3). By using an algorithm-based approach, we have also identified human TSHR peptides with A^k-binding potential and tested their immunogenicity and pathogenicity in AKR/J mice (Chapter 4). Finally, an alternative method for identifying pathogenic Tg epitopes using DNA immunization was introduced and preliminary data are being presented (Chapter 5).

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LIST OF ABBREVIATIONS

a.a.	Amino acid
Ab	Antibody
Ag	Antigen
AITD	Autoimmune thyroid disease
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BGH	Bovine growth hormone
bp	Base pair
cAMP	Cyclic AMP
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CG	Chorionic gonadotropin
CHO	Chinese hamster ovarian
CpG	Cytosine-phosphate-guanine
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagles Medium
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
EAT	Experimental autoimmune thyroiditis
ECD	Ectodomain
e.d.	Epidermal
ELISA	Enzyme-linked-immunosorbent assay
FBS	Fetal bovine serum
GD	Graves' disease
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEL	Hen egg lysozyme
hGH	Human growth hormone
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HT	Hashimoto's thyroiditis
hTg	Human thyroglobulin
hTSHR	Human TSHR
i.d.	Intradermal
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscular

i.p.	Intraperitoneal
ISS	Immunostimulatory sequences
LB	Leria Bertani
LCs	Langerhan's cells
LH	Leuteinizing hormone
LNC	Lymph node cells
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MBP	Maltose binding protein
MCF	Mean channel fluorescence
MHC	Major histocompatibility complex
mRNA	Messenger RNA
mTg	Murine thyroglobulin
mTSHR	Murine TSHR
MW	Molecular weight
NP	Nucleoprotein
OD	Optical density
p2494	mTg peptide (a.a. 2494-2510)
p2964	mTg peptide (a.a. 2964-2711)
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS-T	PBS with Tween
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
rTg	Rat Tg
RT-PCR	Reverse transcriptase PCR
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SD	Standard deviation
T3	Triiodothyronine
T4	Thyroxine
TCR	T cell receptor
Tg	Thyroglobulin
Th	T helper lymphocyte
Th1	T helper 1 lymphocyte
Th2	T helper 2 lymphocyte
TPO	Thyroid peroxidase
TSAb	Thyroid stimulating antibodies
TSBAb	Thyroid stimulating blocking antibodies
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
TBII	TSH-binding inhibitory immunoglobulins

CHAPTER ONE

INTRODUCTION

1.1 Graves' Disease

Graves' Disease (GD) is an autoimmune disorder characterized by lymphocytic infiltration of the thyroid gland and autoantibodies specific for the thyroid stimulating hormone receptor (TSHR) on the membrane of thyroid follicular cells[1]. When these antibodies bind to the TSHR, they stimulate thyroid cells to grow and release thyroid hormones (thyroxine; T4 and triiodothyronine; T3) into the bloodstream. Unlike the natural receptor ligand (thyroid stimulating hormone; TSH), these thyroid-stimulating antibodies (TSAb), are not regulated by a feedback mechanism, and consequently overstimulate the thyroid gland causing hyperthyroidism[2]. High levels of thyroid hormones in GD patients can result in acute weight loss, excessive sweating, a rapid, irregular heartbeat and anxiety. However, not all antibodies specific for the TSHR molecule act as agonists. Patients with primary myxoedema, for example, contain TSHR-specific antibodies (TSBAb) that bind to different sites on the receptor, but block the binding of TSH and cause thyroid atrophy and hypothyroidism[3]. Evidence also exists for neutral TSHR-specific autoantibodies, which neither activate the thyrocyte nor block ligand action[4]. Several studies have shown that GD patients may have both TSAbs and TSBAbs suggesting that the relative amounts or affinities of these two types of autoantibodies determine the net biological effect on thyroid cell function[5]. Cell lines expressing recombinant TSHR have been used to detect the stimulating and blocking activity of these autoantibodies. While TSAbs enhance cAMP production in such cells,

TSBAbs inhibit cAMP production mediated by TSH[6]. Another commonly used assay detects TSH-binding inhibitory immunoglobulins (TBII), comprising both the thyroid-stimulating and TSH-blocking Abs, based on their ability to inhibit the binding of ^{125}I-labelled TSH to its receptor in thyroid membrane preparations[6].

1.2 Structure and Functional Domains of TSHR

Molecular cloning and functional expression of the human TSHR led to rapid advances in understanding the structure and function of this molecule. The TSHR belongs to the guanine nucleotide regulatory (G)-protein-coupled receptor superfamily and is comprised of 764 amino acids (a.a.) organized into a large (398 a.a.) N-terminal extracellular domain (ECD) responsible for interacting with the hormone ligand (TSH), and a C-terminal transmembrane/intracellular region (346 a.a.) responsible for signal transduction[3;6-9]. The protein contains a signal peptide of 20 amino acids which is cleaved during transport to the cell surface (~84.5 kDa)[3;6-9].

The 398 residues of the TSHR-ECD are loosely arranged into ~ 9 leucine-rich motifs potentially forming amphipathic β-sheets[3;6;8] with intramolecular disulphide bonds linking the cysteine residues located at the N- and C-termini of the ECD[9]. Compared to other related G-protein-coupled receptors such as luteinizing hormone (LH) and chorionic gonadotropin (CG) receptors, the ECD of the TSHR contains two unique insertion sites at positions 38-45 and 317-366, making it larger than LH and CG receptors by approximately 60 a.a[3]. Transfection of mammalian cells with various human TSHR deletion mutants or LH/CG receptor chimeras revealed that TSAb bind mainly to the N-terminal region of the TSHR-ECD whereas TSBAb recognize mainly the C-terminal

region of the ECD[7]. Receptor glycosylation also appears to be a strict requirement for high affinity binding of pathogenic autoantibodies indicating that the 6 complex carbohydrate side chains on the TSHR protein may play a subtle role in the correct maintenance of the tertiary structure of the autoantigenic epitopes[7;9].

The 346 residues of the transmembrane region are organized into seven transmembrane segments, three extracellular loops, three intracellular loops, and a short (~ 82 a.a.) carboxyl-terminal cytoplasmic tail[8]. While the importance of the TSHR-ECD for both TSH and TSHR-specific autoantibody binding is well recognized, it remains unclear whether the three extracellular loops linking the seven transmembrane-spanning regions contribute to the high-affinity binding of TSH, or to the binding of some or all the populations of TSHR-specific autoantibodies[7].

1.3 Animal Models of Graves' Disease

In many organ-specific autoimmune diseases, such as Hashimoto's thyroiditis (HT), animal models have contributed considerably to our understanding of the disease process. An animal model of GD must exhibit most of the clinical signs and symptoms of GD including (i) elevated thyroid hormone and/or reduced TSH levels; (ii) antibodies to the TSHR having biological activity (TSAb); (iii) changes in thyroid architecture; and (iv) lymphocytic thyroiditis. Currently, an animal model developing spontaneous GD does not exist. So far, much of the research in this area has focused on experimentally inducing GD in mice.

1.3.1 Immunization with purified TSHR

Initial attempts to immunize mice with purified TSHR were hindered by very low numbers of TSHR expressed on thyrocytes (~10^3-10^4 TSHR molecules/cell)[10], and the instability of the protein[11]. Slightly larger amounts of TSHR were obtained by detergent extraction from the human thyroid cell line (GEJ)[12] followed by TSH-affinity column purification. Repeated immunization of H-2-congenic mouse strains on B10 background with this receptor preparation emulsified in CFA (~ 5 μg/immunization) induced low levels of TBII, mild thyroiditis, and transient increase in free T3 levels in H-2^s, H-2^b, and H-2^q mice[13]. Yet, even with the use of the GEJ cell line and an extensive purification process, limited quantities of antigen were acquired.

1.3.2 Xenotransplantation of PBMCs or T cell lines plus autologous thyroid tissue into SCID mice

An alternative approach to developing an animal model of GD involved transplanting thyroid tissue and immune cells from patients with autoimmune thyroid disease (AITD) into mice with severe combined immunodeficiency disease (i.e. SCID mice). Such mice accept xenografts of human tissue because they lack mature T and B cells[14]. Human IgG class autoantibodies to thyroglobulin (Tg) and thyroid peroxidase (TPO) developed in SCID mice engrafted with suspensions of blood lymphocytes, thyroid lymphocytes, or even intact thyroid tissue from Graves' or Hashimoto patient donors[15-19]. More importantly, some mice xenografted with thyroid tissue from GD patients developed TSHR antibodies and transient hyperthyroxinemia[19]. In spite of the presence of TSHR antibodies, these mice did not show persistent hyperthyroxinemia, presumably because of the destructive thyroiditis observed shortly after tissue grafting. In

attempt to induce a more intense response, Soliman et al.[20] engrafted SCID mice with thyroid tissue from a GD patient together with T cell clones reactive to recombinant hTSHR-ECD and observed low, but detectable levels of TSAb in these mice. The occurrence of these TSAb, however, did not correlate with an increase in serum T3. Greater success was obtained following simultaneous xenotransplantation of tissue and autologous bone marrow cells from GD patients[21]. Higher, but still variable, levels of thyroid autoantibodies, including TSAb, were detected in these mice as well as elevated T4 levels for the 8-week period of the study.

The difficulty and expense of maintaining SCID mice in a sterile environment, the variability between individual mice, as well as the difficulty in obtaining MHC-matched T cell clones, thyroid grafts, and autologous bone marrow cells are major limitations in the use of this approach to develop an animal model for GD.

1.3.3 Immunization with recombinant TSHR

Cloning of the human TSHR gene[22-25] facilitated recombinant TSHR expression and purification from prokaryotic and eukaryotic cells. Several laboratories have stably expressed TSHR in Chinese hamster ovarian (CHO)[25-30] or SP2/0 cell lines[31] for the purpose of generating large amounts of recombinant protein. Although these cells have been shown to transduce a signal after TSH[25;30] and TSHR autoantibody[30;32;33] stimulation, and have been utilized in TSH-binding inhibition assays[32], TSHR expression was still too low to make purification practical. Even with as many as 100, 000 TSHR molecules/CHO cell, only 1 μg of TSHR could be obtained from 6 confluent 10-cm dishes of cells; assuming 100% recovery[6].

Numerous groups have reported on the prokaryotic expression of the TSHR ectodomain (TSHR-ECD)[34-43]. Costagliola and colleagues used *E. coli* to express human TSHR-ECD fused to the maltose binding protein (MBP)[35]. They immunized BALB/c (H-2^d) mice intraperitoneally (i.p.) with this fusion protein emulsified in alum and attenuated *Bordetella pertussis* toxin adjuvant and reported induction of TSBAbs and TBII, as well as mild thyroiditis in this strain[44]. A very mild hypothyroxinemia, based on total T4 values, was observed but it was also evident in control mice that received the MBP fusion partner alone, thus rendering these results inconclusive. Subsequently, the same group showed lack of similar responses in other strains of mice suggesting genetic control in the generation of these antibodies[45]. Thyroiditis was also induced in BALB/c mice following multiple challenge with recombinant TSHR-ECD lacking the N and C-terminus (a.a. 43-282)[43]. The authors suggested that removal of the serological immunodominant epitopes unmasked cryptic, disease-inducing T cell determinants. Despite thyroiditis induction, thyroid function remained unaffected in these mice. A major drawback using receptor produced in prokaryotic cells was the inability of bacteria to produce correctly folded and glycosylated protein[46]. In fact, recombinant protein produced in bacteria was usually denatured and not recognized by either TSH or autoantibodies whose binding critically depends on the correct conformational folding of the receptor polypeptide[33;47].

It was hoped that eukaryotic expression of TSHR in insect cells, using baculovirus vectors, might offer a solution to this problem. Unlike other mammalian G-protein-coupled receptors which have been expressed in high levels in insect cells[48-50], the entire TSHR could not be expressed in insect cells for some unknown reason[27;51-53]. In

contrast, several research groups have expressed the ectodomain using this system[36;42;51;52;54-58]. Indeed, glycosylated human ectodomain produced using this system is superior to the antigen from bacteria in terms of absorbing out TBII activity from GD patients' sera[54;58], and in some cases, TSH binding[54;57]. Immunization of various strains of mice (H-2 $^{b, d, k, s}$) with human TSHR-ECD produced by this method resulted in high titers of antibody against the TSHR protein which were capable of blocking the binding of TSH to native TSHR, but lacked biological activity[59]. Interestingly, elevated thyroxine levels were observed in BALB/c mice and, despite the absence of thyroiditis, there were morphological changes in the gland indicative of hyperactivity, such as focal scalloping of the colloid within isolated acini[59]. Another group showed that similarly challenged CBA/J mice also elicited TBII recognizing TSHR amino acids 22-41 as well as peptides clustered toward the carboxyl-terminus of the hTSHR-ECD (a.a. 322-415)[60]. However, unlike BALB/c mice, CBA/J mice remained euthyroid, and thyroid histology was normal. Carayanniotis, et al.[61] reaffirmed the immunogenicity of insect-cell derived hTSHR-ECD in five different strains of mice (H-2$^{b, d, q, k, s}$) by eliciting strong specific T cell responses and IgG responses in all strains following subcutaneous challenge with antigen emulsified in CFA. TBII were only detected in mice subjected to a hyperimmunization regime correlating with previous findings[60]. Unlike Wagle et al.[59] who observed increased serum T4 levels and changes in thyroid histology of BALB/c mice, alterations in thyroid function as assessed by serum TSH, T4, iodine levels, or thyroid architecture were not detected in BALB/c mice or any other mouse strain. Suspecting that the inability to induce hyperthyroidism in mice was related to use of a heterologous rather

than homologous antigen, Davies' group expressed the biologically active homologous mTSHR-ECD in insect cells[62]. Indeed, sequencing of the mTSHR[63] revealed potentially important differences from the hTSHR sequence, which could alter the tertiary structure of the receptor and prevent or diminish induction of agonist antibodies. The fact that only a minority of hTSHR-specific antibodies that bind to the mTSHR, act as TSH agonists also agrees with this interpretation[64-66]. When BALB/c mice were challenged with murine TSHR-ECD in alum adjuvant containing pertussis toxin, TBII and TSBAb were induced, and mice had reduced levels of T3 accompanied by increased TSH, but no signs of thyroiditis or thyroid destruction were observed[67]. A later study compared the immunogenicity of extracellular domains of glycosylated mouse and human TSHR[68]. Mice immunized with either ectodomain developed high titers against their respective immunogens and comparable titers specific for TSHR peptides 1 (a.a. 22-41) and 23 (a.a. 352-371). TBII reactivity was slightly higher in mice immunized with human TSHR-ECD than murine TSHR-ECD (52.2 % vs. 34.5 %), while T4 levels from murine TSHR-ECD immunized mice were marginally higher than controls. Overall, the outcome of immunization with mouse TSHR-ECD was comparable to that seen after immunization with human TSHR-ECD.

Despite all efforts, none of the above studies induced mouse hyperthyroidism in a consistent and reproducible manner. The apparent variation and discrepancies in the results can be attributed to many factors including the adjuvant used, route and frequency of antigen administration, the TSHR form (truncated or combined to a fusion protein), lack of the transmembrane region, differential glycosylation and inappropriate folding of

the receptor. Altogether, the data argued strongly in favor of using the native form of TSHR for the production of agonist Abs.

1.3.4 Immunization with synthetic TSHR peptides

Several studies have reported induction of TBIIs, TSAbs, and TSBAbs in mice, rabbits, and birds immunized with synthetic TSHR peptides corresponding to various regions of the TSHR molecule including: a) the unique insertion sequences of the TSHR molecule (a.a. 38-45 and 317-366); b) potential TSH-binding sites (a.a. 372-397 and 341-358); and c) extracellular loops from the transmembrane region (a.a. 478-497, 561-580, 649-652)[69-74]. Surprisingly, only two of these studies measured thyroid hormone levels[72;74], one of which mentioned an increase in T3 and T4 levels that correlated with TSAb activity following immunization with the unique N-terminal region (a.a. 29-57) of the hTSHR[74]. These results imply that linear epitopes can induce production of TSAbs, contradicting the general concept that TSAb only recognize conformational epitopes[33;47]. This interpretation, however, has to be confirmed since this group did not attempt to block the functional activity of TSAbs with the immunizing antigen. This is particularly important since immunization with some peptides can cause the breakdown of tolerance to self-TSHR, resulting in a much broader immune response than the one against the immunizing peptide[3]. Nevertheless, the above observations as well as the fact that a great majority of GD patient sera recognize linear TSHR epitopes do not exclude the possibility that linear epitopes recognized by TSAb exist.

1.3.5 Immunization with fibroblasts or B cells stably expressing native TSHR

To achieve native expression of TSHR, Shimojo et al.[75] transfected the RT4.15HP murine fibroblast cell line (L cells) expressing MHC class II molecules (A^k) with the full-length human TSHR. They challenged AKR/N ($H\text{-}2^k$) mice intraperitoneally six times every 2 weeks with 10^7 mitomycin-C-treated fibroblasts and reported thyroid enlargement, TSAbs, and increased T4 levels in 20% (5/24) of experimental mice. Histological examination of the thyroids revealed hypercellularity but no lymphocytic infiltration. In a second series of experiments, the non-MHC genetic control of the induced disease was investigated in five different $H\text{-}2^k$ strains of mice[76]. Differences in TBII titers were observed between strains indicating non-MHC genetic effect on TSHR-Ab formation. Surprisingly, CBA and C3H strains had circulating TBII following challenge with fibroblasts expressing TSHR only, but this was not associated with development of hyperthyroidism. Elevated T4 levels were only observed in mice immunized with fibroblasts expressing both MHC class II and hTSHR suggesting that aberrant class II expression is necessary for the development of GD, whereas non-MHC genes play a more limited role. In 1999, Kita et al.[77] and Jaume et al.[78] independently confirmed and extended Shimojo's original findings using similarly transfected RT4.15HP fibroblasts. In the former study, most animals developed TBII, and elevated T4 levels were induced in 5/20 (25 %) recipients of fibroblasts expressing receptor and MHC class II. One mouse had elevated TSH and reduced T4 levels but the thyroid was normal. The five animals with increased T4 had enlarged thyroids with signs of hypertrophy and colloid droplet formation but no lymphocytic infiltration. Mice

immunized using the same protocol, but with the addition of a Th2 adjuvant (alum and pertussis toxin) had an earlier onset of disease (9 weeks compared to 11 weeks), and elevated T4 and goiters were found in a larger proportion of the mice (47 % compared to 25 %). In contrast, i.p. injection of AKR/N mice with hTSHR$^+$ and MHC class II$^+$ fibroblasts together with a Th1 adjuvant (CFA), resulted in a slower onset of hyperthyroidism with 10/31 mice having increased T4 by 14 weeks. Like many autoimmune diseases, GD occurs predominantly in women[1], yet Jaume et al.[78] observed no difference between male and female AKR/N mice in terms of susceptibility to TSHR antibody induction or development of hyperthyroidism following injection of TSHR$^+$, MHC class II$^+$ fibroblasts.

The mechanisms behind the induction of hyperthyroidism using transfected fibroblasts in these initial studies were not understood. For instance, how was the antigen processed and presented? Fibroblasts cannot act as APC initiating the immune response since they lack B7-1/B7-2 co-stimulatory molecules required for T cell activation[79;80]. Were thyroid antigens shed from dying fibroblasts and presented by conventional host APCs? Why is aberrant expression of MHC class II required for disease induction? Why was disease induced in only 20-30% of the animals? Finally, why does this model, like many others, fail to induce intrathyroidal lymphocytic infiltrate? Clearly, the mechanisms underlying Shimojo's model have to be further investigated. Kaithamana et al.[80] used a variation of this approach by immunizing BALB/c mice with a B lymphoblastoid cell line (M12; derived from BALB/c mice) stably transfected with either murine TSHR (mM12) or human TSHR (hM12). They reported disease induction characterized by the presence

TBII, TSAb, elevated thyroid hormone levels (T3 and T4), and weight loss in almost 100% of the mice. Hyperthyroid mice had enlarged thyroid glands relative to control thyroids, and histological examination revealed hypertrophy, enlarged colloids with thinning epithelium, and in some cases, focal necrosis and inflammation characterized by lymphocytic infiltration. The authors attributed the greater success rate to several factors including the expression of B7-costimulatory molecules on M12 cells making them more efficient APC *in vivo*, the different and potentially more susceptible mouse strain used, and native expression of the MHC class II molecules. However, B7 expression on M12 cells was not confirmed in this study, and in other studies, M12 cells were shown to lack B7 co-stimulatory molecules[81]. Regardless of B7 expression or lack thereof, M12 cells transfected with either the human or murine TSHR induced hyperthyroidism in almost 100 % of experimental mice. Surprisingly, a later study performed by Yan, et al.[79] showed that RT4.15HP fibroblasts do indeed express B7-1 co-stimulatory molecules, which may be associated with the widespread immune activation observed in AKR/N mice when challenged with RT4.HP15 fibroblasts alone. Initially, the goal of the experiment was to determine whether the lack of thyroid lymphocytic infiltration in AKR/N mice was related to an inability of this strain to develop a Th2 response following injection with $TSHR^+$, MHC class II^+, RT4.15HP fibroblasts. While attempting to characterize the Th1/Th2 cytokine profile through IgG subclass analysis of TSHR-specific antibodies, Yan et al.[79] found that recipients of RT4.15HP fibroblasts, whether transfected or not with the TSHR, displayed high-levels of non-specific binding to TSHR-coated ELISA wells or to TSHR-expressing cells. Moreover, splenocytes from all

fibroblast-injected (but not from unimmunized) AKR/N mice spontaneously secreted high levels of interferon-γ *in vitro*. These results indicate that B7-1$^+$, RT4.15HP fibroblasts have the potential to act as APC and may be responsible for the initiation of the immune response to the TSHR, as well as the non-specific immune activation. Alternatively, the widespread immune activation may be caused by an alloreactive response against the chimeric class II β1 domain derived from H-2^{d} [82] as suggested by Kaithamana et al.[80] since alloantigens are more potent immunogens than self Ags.

Shimojo's approach for establishing a mouse model for GD succeeded in reproducing major features of GD including elevated thyroid hormone levels, TSAb activity, goiter, and minimal lymphocytic infiltration. However, the lack of similar derived fibroblasts expressing class II molecules from other mouse strains limits investigation to mice with an H-2^{k} haplotype. Kaithamana et al.[80] were able to immunize BALB/c mice, a different mouse strain with an H-2^{d} haplotype, only because they used a B cell line derived from BALB/c. It would be interesting to see whether a non-specific immune response also occurred in these mice.

1.3.6 Genetic immunization with hTSHR cDNA

In 1998, genetic immunization with a human TSHR cDNA construct in a mammalian expression vector mounted a very strong immune response in BALB/c mice[83]. Anti-TSHR antibodies recognizing the native TSHR were readily observed in all mice, and both TBII and TSBAb activities were present in their serum. However, in the only instance in which it was observed, TSAb activity was not associated with hyperthyroidism. Convinced that the failure to develop hyperthyroidism was related to an

inadequate genetic background, the same group proceeded to genetically immunize NMRI outbred mice[84]. This time, five out of 29 female mice showed signs of hyperthyroidism including elevated total T4 and suppressed TSH levels correlating with TSAb activity. In addition, hyperthyroid animals had goiters with extensive lymphocytic infiltration and displayed ocular signs reminiscent of Graves' ophthalmopathy. In spite of these encouraging results, less than 20% of the animals developed hyperthyroidism, which the authors suggested was due to the outbred nature of NMRI mice, thus providing no further information in regards to the genetic requirement for susceptibility. Another group[85] recently used a similar DNA immunization regime in BALB/c mice and obtained results conflicting with those of Costagliola's et al.[83;84]. Mice vaccinated with hTSHR cDNA had low or undetectable levels of TSHR antibodies as determined by ELISA or flow cytometry and virtually no detectable TBII or TSAb. Moreover, thyroid histology revealed no architectural changes or lymphocytic infiltration[85]. The underlying mechanisms involved with DNA immunization, need to be further investigated to enable the establishment of a consistent and reproducible animal model of GD.

1.4 Genetic Immunization

Genetic immunization, also known as DNA or polynucleotide immunization is a novel approach for achieving both humoral and cellular immune responses. The concept behind genetic immunization is simple: genes encoding an antigen (or antigens) specific for a particular pathogen are cloned into a plasmid with an appropriate promoter, and the plasmid DNA is administered to the vaccine recipient. Host cells take up the DNA and express the encoded protein, which is then processed and presented to the immune

system, inducing a specific immune response. The efficacy of DNA immunization was first demonstrated in 1992 when Tang et al.[86] reported that inoculation of mice with gene gun-delivered plasmid DNA induced antibody responses to the encoded human growth hormone (hGH) protein. Shortly thereafter, DNA vaccines were shown to generate immune responses against influenza[87;88], human-immunodeficiency virus (HIV)-1[89], and hepatitis B[90]. These earlier studies not only demonstrated that DNA vaccination could induce immunity in several different disease models, but also that antibody, T-helper (Th), and cytotoxic T-lymphocyte (CTL) responses could all be generated through various immunization routes (i.e. intramuscular, epidermal, or mucosal). Furthermore, in the case of influenza, immunization with plasmid DNA encoding nucleoprotein (NP) of influenza A could protect animals from subsequent challenge with pathogenic virus. DNA vaccine development against other infectious disease models including herpes-simplex virus (HSV)-1[91], rabies[92], malaria[93], and mycoplasma[94] are currently under investigation. The generation of immune responses through genetic immunization has not been limited solely to viral, bacterial, and parasitic diseases. Injection of plasmid DNA encoding tumor-associated antigens has been shown to cause a tumor-antigen-specific immune response, and can protect mice from subsequent lethal tumor challenge[95;96]. In addition, numerous studies have prevented or suppressed experimental or spontaneous autoimmune disease induction in mice following immunization with DNA encoding the appropriate self-antigen[97-101]. The list of potential applications for DNA immunization has generated considerable excitement in the vaccine field, especially for pathogens such as HIV, *Plasmodium falciparum*, *Mycobacterium tuberculosis*, for which no conventional

vaccines are available. However, before it is tested in humans, the underlying mechanisms must be understood (Section 1.4.1), immune responses need to be optimized (Section 1.4.2), and the safety concerns (Section 1.4.3) need to be addressed.

1.4.1 Mechanisms of immunity induced by DNA immunization

Although a remarkable number of publications have reported the efficacy of DNA vaccination in animal models, the exact mechanism by which the antigen encoded by the foreign gene introduced into the bacterial plasmid is processed and presented to immune system remains unclear. Since DNA vaccination has been shown to induce both humoral and cell-mediated immune responses, each of the three arms of the immune system must encounter the encoded antigen in the appropriate context. B cells should recognize and bind to soluble antigen, $CD4^+$ T cells should recognize peptide-MHC class II complexes on the surfaces of APCs that have endocytosed and processed exogenous antigens, and $CD8^+$ T cells should recognize peptide-MHC class I complexes derived from endogenously produced protein that has undergone proteosome-dependent-intracellular processing. Three mechanisms have been proposed to explain how the antigen encoded by plasmid DNA is processed and presented to immune cells: a) direct priming by somatic cells that have taken up DNA; b) direct transfection of professional APCs; and c) cross-priming[102;103]. Since intramuscular (i.m.) DNA immunization primarily transfects myocytes[104]; it was originally thought that these cells act as antigen-presenting cells. Myocytes, which constitutively express class I molecules[105], could induce $CD8^+$ CTL responses through the continuous processing of plasmid-derived antigen, whereas soluble protein, either secreted from myocytes or released upon myocyte death, could generate

antibody responses and T cell help. One conceptual difficulty with this premise is that, myocytes lack important co-stimulatory molecules necessary for priming naïve T cells[106]. The second hypothesis proposes that a small number of professional APCs are directly transfected with the injected DNA. These cells, expressing high levels of MHC class I, as well as co-stimulatory molecules, then traffic to regional lymphoid tissue, where they can activate $CD8^+$ T cells. Meanwhile, $CD4^+$ T cell and humoral immune responses are generated by soluble antigen produced by somatic cells or APCs. This mechanism seems plausible following epidermal (e.d.) or intradermal (i.d.) immunization, because the skin is rich in potential APCs including Langerhans cells (LCs), and tissue macrophages[107]. However, it seems less likely to occur following i.m. immunization, since muscle contains few if any APCs[105], although some professional APCs may be recruited to the muscle by the local irritation that follows injection. Finally, the cross-priming hypothesis suggests that antigen produced by transfected myocytes or keratinocytes is transferred to bone-marrow-derived APCs that have infiltrated the muscle as part of an inflammatory response to the immunization procedure. The internalized protein would then either be processed through the endocytic pathway resulting in peptide-MHC class II complexes, or will 'cross over' into the MHC class I pathway allowing the APC to activate both $CD4^+$ and $CD8^+$ T lymphocytes. Although the cross-priming theory contradicts the dogma that only endogenously produced proteins can enter the MHC class I pathway, recent reports have described how exogenously produced protein can be taken up by APCs and then presented in the context of MHC class I molecules[106;108-111].

Various experiments were undertaken to directly test whether muscle cells alone were sufficient to prime CTL immune responses or whether bone marrow-derived APCs were required for CTL priming. Using bone marrow chimeric mice and plasmid DNA encoding the influenza nucleoprotein (NP), Corr and colleagues[112], demonstrated that NP-specific CTL responses were restricted to the MHC haplotype of the donor bone marrow-derived cells and not by the haplotype expressed by the recipient's myocytes. Similar findings for i.m. immunization were reported by Doe et al.[113] and for e.d. gene gun immunization by Iwasaki, et al.[114] The latter group also showed that even if myocytes were co-transfected with a plasmid encoding co-stimulatory molecules along with the influenza NP plasmid, they were still unable to prime CTL responses. These results, however, contradict those obtained by Agadjanyan et al.[115] where Ag-specific CTL responses could be induced by muscle cells when mice were co-injected with DNA encoding Ag and CD86. Nevertheless, these studies strongly support the requirement of APCs for priming CTL responses, and that nonhematopoietic cells such as myocytes or keratinocytes are not being converted into APCs by naked plasmid transfection.

The exact mechanism by which bone marrow-derived APCs obtain antigen, however, remains under considerable debate. Evidence supporting direct transfection of APCs by plasmid DNA comes from a study by Casares, et al.[116] who were able to isolate plasmid DNA from lymph node-derived and skin-derived dendritic cells after i.m. and i.d. immunization respectively. Further support comes from the finding that removing the muscle immediately (within 10 minutes) after immunization does not alter the subsequent immune response indicating that the role played by myocytes as an 'antigen factory' is

minimal[104]. Other research groups, however, argue against the 'direct transfection' hypothesis, and have obtained evidence supporting the 'transferred antigen' hypothesis. Ulmer and colleagues[117], for example, were able to induce both anti-NP Abs and NP-specific CTL responses in mice previously transplanted with myoblasts stably transfected with influenza NP DNA. As there was obviously no free plasmid available to directly transfect specialized APCs, these studies strongly suggest that antigen expressed by the transfected myoblasts was transferred to APCs and processed through the cytosolic MHC class I pathway. Similar findings were also found following transplantation of transfected tumor cells[109;118]. The strong evidence supporting both theories implies that both mechanisms probably play a role in the generation of immunity through genetic immunization, or alternatively, other mechanisms not yet discovered, may be involved.

1.4.2 Approaches to DNA vaccine optimization

Different diseases have specific requirements for protective immunity; thus a rational approach to optimize DNA vaccination would reflect these distinct conditions. A key advantage that DNA vaccination has over conventional vaccines is the ease with which plasmid DNA can be manipulated to alter the quantitative and qualitative aspects of an immune response. Simple vector modifications, supplementing Ag-encoded vectors with vectors encoding cytokines and co-stimulatory molecules, altering plasmid DNA dose or mode of DNA administration, can easily enhance and/or redirect the immune response which benefits the recipient most.

One of the most important consideration in optimizing a DNA vaccine is the appropriate choice of a vector. The essential features for a plasmid DNA vector include:

a) a strong viral promoter active in mammals (i.e. cytomegalovirus) for optimal Ag expression; b) an insert encoding the Ag; and c) a termination sequence (i.e. BGH poly A tails) that stabilizes mRNA transcripts[103]. In addition, DNA vaccines require 'cytosine-phosphate-guanine (CpG) motifs' or immunostimulatory sequences (ISS) known to impart adjuvanticity and mitogenicity to plasmid DNA. This CpG motif/ISS consists of an unmethylated cytosine-phosphate-guanosine dinucleotide with appropriate 5' and 3' flanking regions. Such motifs are 20-fold more common in microbial than mammalian DNA and have been shown to directly activate B cells to proliferate and produce Ab[119], induce various APCs to secrete cytokines[120-122] that indirectly activate natural killer (NK) cells, and directly or indirectly stimulate T cells[123]. The immunogenicity of CpG motifs became relevant when Sato et al.[124] reported that a DNA vaccine whose plasmid backbone contained CpG ISS induced a more vigorous antibody and CTL response than an otherwise identical vaccine lacking ISS, despite a higher level of gene expression produced by the latter plasmid. Thus, reengineering plasmid backbones to contain additional CpG motifs is one way to enhance the immune response against the encoded Ag. Interestingly, ISS that induce optimal stimulation in mice are less effective when tested on cells of primate origin. Attempts to identify sequence motifs that are equally immunogenic in humans have, so far, revealed two different CpG motifs capable of stimulating different cell populations[125;126]. These results suggest that a wide variety of motifs exist, each of which may target and stimulate different immune cells. Thus, selectively engineering DNA vaccines to contain one or various stimulatory sequences may help tailor the type of immune response elicited against the encoded Ag.

Cytokine and co-stimulatory DNA adjuvants have also been shown to enhance or bias the immune response generated by DNA vaccination (reviewed by Gurunathan, et al.[103]). This is not surprising since cytokines and co-stimulatory cell surface molecules play a crucial role in generation of effector T-cell subsets and in determining the magnitude of the response. Co-administration of DNA immunogens (e.g. hepatitis C virus core protein, HIV envelope protein, or ovalbumin) with either IL-2 or IL-4 cytokine genes, for example, has been shown to enhance T cell proliferative responses to the respective protein antigen *in vitro*, with cytokine profiles corresponding to Th1 and Th2 phenotypes respectively[127-132]. Enhancement of overall antibody production in mice was also demonstrated, with a bias toward IgG2a and IgG1 isotypes by co-injection with IL-2 and IL-4 DNA expression constructs respectively[127-132]. Similarly, intramuscular injection of a B7-2 gene expression cassette along with cassettes for influenza nucleoprotein[133] or HIV proteins[134] results in a significant enhancement of cytotoxic T cells responses to the encoded Ag in mice.

Successful DNA vaccinations have been demonstrated via a number of different routes including: i.m., i.d., e.d., intravenous (i.v.), intraperitoneal (i.p.), oral, intranasal, and vaginal. Not only does the site of inoculation affect the induced immunity, but also the method the plasmid is delivered can qualitatively affect the immune response. Plasmid delivery is predominantly accomplished by one of two methods: needle injection of DNA suspended in saline or in a saline mixture containing a facilitator, such as bupivacaine, designed to enhance DNA uptake[102]; or propulsion of DNA-coated-microprojectiles (gold beads) directly into the target tissue using a gene-gun. In terms of

DNA dosage, gene-gun-mediated immunization is far more efficient than needle injection since similar levels of Ab and cellular responses are elicited with 100- to 5000-fold less DNA[89;135;136]. Nevertheless, there has been no compelling evidence to indicate that gene-gun-based immunizations lead to longer lived responses or greater protection from pathogenic challenge. However, the way a DNA vaccine is administered can affect the T helper cell profile that is ultimately generated. While i.m. needle injection of DNA produces a predominantly Th1-type response, with an elevated IgG2a:IgG1 ratio, IFN-γ production, and little IL-4 production[135;137;138], successive e.d. gene gun inoculations generate mostly IgG1 antibodies, less IFN-γ, and more IL-4, indicative of a Th2-type response[135;137;139]. Induction of both Th1[135;140] and Th2[137] profiles has been reported following i.d. injection of DNA. It remains unclear why different T helper subsets arise after different immunization regimes; however, one possibility is a dosage phenomenon. As mentioned above, needle injections require larger quantities of DNA than gene-gun inoculations to induce the same magnitude of an immune response. Larger amounts of DNA introduce significantly more ISS to the challenge site, and ISS have been shown to preferentially induce a type 1 helper T lymphocyte response[121;124;141;142]. Experimental evidence supports the claim that when a few micrograms of DNA are used, delivery by both gene gun and i.m. needle injections induces predominantly IgG1 Abs, indicative of a Th2 response[143], but when 50 μg of DNA are used both methods induce more IgG2a Abs, indicative of a Th1 response. Other studies, however, found that as little as 1-2 μg of DNA after i.m. injection, still elicits IgG2a Abs, while equivalent doses using a gene gun elicit predominantly IgG1 Abs[135]. Alternatively, different ways of DNA uptake by

surrounding tissue cells may affect the differentiation of the two Th subsets following needle or gene gun DNA administration. Needle injection delivers DNA to the extracellular space, where it is taken up by cells by some poorly understood mechanism, perhaps inducing a set of intracellular signals that ultimately leads to the increased production of Th1-type cytokines. A different set of intracellular signals, however, may be generated following gene gun delivery since target cells are directly transfected with plasmid, and therefore bypass any uptake mechanisms[102].

1.4.3 Safety consideration of DNA vaccination

DNA vaccines offer several advantages over many of the existing vaccines. For example, plasmid DNA: a) is easier to manufacture b) obviates the need for protein production and purification, and c) circumvents the use of external adjuvants because plasmids impart their own adjuvanticity (e.g. ISS). In addition, immunization with plasmid DNA induces both humoral and cellular immune responses, which can easily be enhanced or manipulated through co-delivery to cytokine-encoding vectors or co-stimulatory molecules, and because DNA immunization provides prolonged Ag expression, immunological memory is generated. All of these advantages, however, coincide with an equal number of safety concerns described in detail by Klinman, et al.[144]. Briefly, potential risks include: a) plasmid integration into the host genome, thereby increasing the risk of malignancy (by activating oncogenes or inactivating tumor suppressor genes); b) induction of responses against transfected cells or worse, against host DNA, thereby triggering the development of autoimmune diseases; c) induction of tolerance rather than immunity; and/or d) non-specific stimulation of cytokine production

that could alter the host's ability to respond to other vaccines and resist infection. Clearly, these safety issues need to be addressed and evidence disproving these risks is required before DNA vaccination can be introduced to the general population.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS

Female BALB/cJ (H-2^d) or AKR/J (H-2^k) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and used for immunizations between 6-12 weeks of age.

2.2 TISSUE CULTURE AND CELL LINES

All tissue culture media including Ham's F12, RPMI-1640, and DMEM (GibcoBRL, Burlington, ON, Canada) were supplemented with 10% fetal bovine serum (FBS) ((Bioproducts for Science, Indianapolis, IN, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco). RPMI-1640 and DMEM were also supplemented with 10 mM Hepes buffer and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO, USA).

CHO-K1, JP09, CHO1, and GPI9-5 cells were derived from the Chinese hamster ovarian (CHO) cell line, and were cultured in Ham's F12 complete medium. They were kindly provided by Dr. J.P. Banga (King's College School of Medicine). JP09 and GPI9-5 cells are CHO cells stably expressing native hTSHR (90 000 hTSHR/cell)[145] and GPI-anchored hTSHR-ECD (unpublished) respectively, while CHO1 cells are vector-transfected control cells. JP09, CHO1, and GPI9-5 cells were positively selected every 10 to 12 passages, using 200 μg/ml of Geneticin (G418) (GibcoBRL, Burlington, ON, Canada). Trypsin-EDTA mixture (GibcoBRL, Burlington, ON, Canada) was used for

routine passaging of CHO cells, while the non-enzymatic cell dissociation medium (Sigma Chemicals, St. Louis, MO, USA) was used to harvest cells prior to experiments.

The IL-4 dependent cell line, CT.4S[146] was a kind gift from W.E. Paul (National Institute of Allergy and Infectious Diseases) and was courteously provided by Dr. T Watts (University of Toronto). It was cultured in RPMI-1640 containing 10 ng/ml recombinant interleukin-4 (PeproTech, Inc., Rocky Hill, NJ, USA). The A10 hybridoma formed between spleen cells from C57/BL6 mice previously immunized with purified insect cell-derived TSHR-ECD and the non-immunoglobulin-secreting myeloma cell line X63-Ag8.653[147] was kindly provided by Dr. J. P. Banga (King's College School of Medicine). The A10 mAb (IgG2b), known to recognize the epitope formed by a.a. 22-35 of the hTSHR was purified from culture supernatants by affinity chromatography on protein G-Sepharose 4 Fast Flow columns (Pharmacia, Baie D'Urfe, Quebec, Canada). The purified material was then dialyzed in PBS, concentrated to 1-2 mg/ml, filter sterilized, and stored at –20°C in 50% glycerol for future use.

The IL-2-dependent cell line, CTLL-2[148], was grown in DMEM supplemented with 10% supernatant from concanavalin-A activated rat splenocytes as a source of IL-2. The antigen-presenting cell line LS 102.9[149], a B-cell hybridoma expressing H-2$A^{d/s}$ and H-E^{d} and the CTLL-2 cell line were purchased from ATCC (Rockville, MD, USA). The TA3 cell line, produced by the fusion of B cells from CAF_1 mice with the M12.4.1 BALB/c B lymphoma[150], was a kind gift from L. Glimcher (Harvard Medical School, Cambridge, MA), and courteously provided by Dr. T. Watts (University of Toronto). TA3 cells are known to express H-2$A^{k/d}$, $E^{k/d}$, $K^{d}D^{d}$ molecules[150]. The T cell hybridomas

4A12.1 (A^k-restricted), 4A2.3 (E^k-restricted), 5E8.6 (A^s-restricted), and 6E10 (A^s-restricted) recognizing murine thyroglobulin (mTg) peptides: p2494 (4A12, 4A2, and 5E8) and p2694 (6E10) were generated and characterized as previously described[151;152].

2.3 MOLECULAR CLONING OF MURINE TSHR

2.3.1 RNA Extraction from Mouse Thyroid Tissue

Two 13-week old BALB /cJ mice were euthanised using ether and their thyroid glands removed *en bloc* (still attached to the trachea). The thyroid lobes were teased away from the trachea and immediately placed in 0.8 ml of TRIzol Reagent (GibcoBRL, Burlington, ON, Canada) containing 250 µg/ml of glycogen (Ambion, Austin, TX, USA). The tissue was then homogenized using a micro-tissue grinder and centrifuged at 12 000 x g for 10 minutes at 4°C to remove insoluble material. The homogenate was then separated into three phases following the addition of 0.16 ml chloroform (Molecular Biology Grade, Sigma, St. Louis, MO, USA) and brief centrifugation (12 000 x g for 15 minutes at 4°C). RNA remains exclusively in the upper aqueous phase. This phase was placed into a new diethyl-pyrocarbonate (DEPC)-treated microcentrifuge tube and RNA was precipitated following the addition of 0.4 ml of isopropanol (Molecular Biology Grade, Sigma, St. Louis, MO, USA) and another brief centrifugation (12 000 x g for 10 min at 4°C). The resultant pellet was then washed with 1 ml of 75% ethanol, air dried, and resuspended in 20 µl DNase-RNase-free water (Sigma Chemicals, St. Louis, MO, USA). The purified RNA absorbance was read using a Beckman Du® 64 spectrophotometer (Fullerton, CA, USA) at the optical density of 260 and 280 nm to determine RNA yield and purity.

2.3.2 First-strand cDNA Synthesis

First-strand cDNA synthesis was performed using a kit from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden). Briefly, 5 μg of purified RNA (made up to a final volume of 20 μl with DEPC-treated water) was placed in an Eppendorf tube and denatured in a Perkin-Elmer™ thermo-cycler (Cetus, Norwalk, CT, USA) at 65°C for 10 minutes, then chilled on ice. The RNA was then added to a pre-mixed solution containing 11 μl first-strand bulk mix, 1 μl dithiothreitol (DTT) solution, 1 μl Not I-d$(T)_{18}$, all provided with the First-strand cDNA synthesis kit. The resultant mixture was then incubated for 1 hour at 37°C. Heating the tube at 75°C for 10 minutes terminated the reaction and the cDNA was stored at –20°C for future use.

2.3.3 Amplification of mTSHR cDNA and Subcloning into pcDNA3.1zeo $^{+}$ Mammalian Expression Vector

The entire mTSHR cDNA insert (2295 bp) was amplified from first-strand cDNA by the polymerase chain reaction (PCR) using mTSHR specific primers. The sequences of the two oligonucleotide primers were as follows (5' → 3'): mTSHR-F = GCC G<u>GA ATT C</u>***AT G***AG GCC AGG GTC CCT and mTSHR-R = C<u>GG CGG CCG C</u>***TT A***CA AGG CTG TTT GCT (GibcoBRL, Burlington, ON, Canada). These primers included the native translation start and stop codons (bold and *italicized*) as well as unique *EcoRI* and *NotI* restriction sites (<u>underlined</u>) outside of the 5' and 3' coding regions (bold) respectively to facilitate subcloning. DNA amplification was carried out using deoxynucleotide triphosphates (dNTPs) from Promega (Madison, WI, USA) and Platinum *Taq* DNA Polymerase (GibcoBRL, Burlington, ON, Canada). Briefly, 1 μl of

cDNA was added to a PCR cocktail containing 1x PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 200 µM of both forward and reverse primers, and 2.5 U of platinum *Taq*. The resultant mixture was then layered with 50 µl of mineral oil (Sigma Chemicals, St. Louis, MO, USA) and placed in the Perkin Elmer DNA thermocycler (Cetus, Norwalk, CT, USA). A 35-cycle step program (denaturation = 94°C for 1 min, annealing = 55°C for 1 min, and extension = 72°C for 2.5 min) was preceded by a 5 minute denaturation step at 94°C and was followed by a 10 minute extension step at 72°C. The amplified PCR product was subject to electrophoresis on 0.7 % agarose gel, and the 2.3 kb fragment was purified using CONCERT™ Rapid Gel Extraction System (GibcoBRL, Burlington, ON, Canada). This fragment was then digested with *EcoRI* and *NotI* (Promega, Madison, WI, USA) and subcloned into a similarly digested and purified pcDNA3.1zeo$^+$ mammalian expression vector (Invitrogen, Carlsbad, CA, USA) using T4 DNA ligase (GibcoBRL, Burlington, ON, Canada). Ligation reactions were diluted 5-fold in nuclease-free water (Sigma Chemicals, St. Louis, MO, USA) prior to transforming bacteria.

2.4 MOLECULAR CLONING OF HUMAN TSHR

The plasmid, pcNeo, containing the human TSHR cDNA insert (pcNeo/hTSHR) between *XhoI* (5') and *NotI* (3') restriction enzyme sites was kindly provided by Dr. J.P. Banga (King's College School of Medicine). The hTSHR cDNA insert was cut from pcNeo/hTSHR and pasted into pcDNA3.1zeo$^+$ mammalian expression vector forming pcDNA3.1zeo$^+$/hTSHR as follows. First, the hTSHR insert was released from pcNeo/hTSHR by cutting the plasmid with *XhoI* creating 5' and 3' sticky ends, which, after purification, were filled in creating blunt ends. Next, the hTSHR insert was

completely released from pcNeo/hTSHR by digesting with *NotI*. Meanwhile, the pcDNA3.1zeo$^+$ mammalian expression vector was cut with *BamHI* resulting in 5' and 3' sticky ends, which, after purification were filled in creating blunt ends. Next the pcDNA3.1zeo$^+$ vector was digested with *NotI*, purified and used to ligate with the purified hTSHR cDNA. During the cutting and pasting of the vector and hTSHR insert, a *XhoI* site was created in the 5' flanking region of the insert, which, together with the downstream *XhoI* site of the vector enabled the release of the hTSHR insert from pcDNA3.1zeo$^+$ with *XhoI*.

2.5 AMPLIFICATION OF MURINE THYROGLOBULIN cDNA SEGMENTS ENCODING PATHOGENIC T-CELL EPITOPES AND SUBCLONING INTO pcDNA3.1zeo $^+$

Total thyroid RNA was extracted from two 21-week old AKR/J mice as described in Section 2.3.1, and first-strand cDNA was synthesized as described in Section 2.3.2. Complementary DNA segments encoding murine Tg pathogenic T cell epitopes, p2494 (2494-2510; GLINRAKAVKQFEESQG) and p2694 (2694-2711; CSFWSKQUQTLKD ADGAK), or the carboxyl terminus of the murine Tg (2494-2746; mTg-C), were amplified from first-strand cDNA by the polymerase chain reaction using Tg-specific primers. Amino acid coordinates of the peptides were assigned according to Kim et al.[153] and do not include the 20 a.a. leader sequence. The sequences of the oligonucleotide primers were as follows (5' → 3'): p2494-KpnI-F = CGG GTA CCG CC*A TG*G GGC TTA TCA ATA G, p2494-XbaI-R = CGT CTA GA *T CA*G CCT TGG CTC TCT T , p2694-KpnI-F = CGG GTA CCG CC*A TG*T GCT CCT TCT GGT, p2694-XbaI-R = CGT CTA GA*T CA*T GCA TCC TTG GCT C , mTg-XbaI-R = CGT CTA GA*T CA*T

TTG CTG TAG CTC TTG (GibcoBRL, Burlington, ON, Canada). Artificial start and stop codons (bold and *italicized*) as well as unique *KpnI* and *XbaI* restriction sites (underlined) were introduced in the forward and reverse primers respectively, outside the 5' and 3' coding regions (bold), to promote translation of the peptides and facilitate subcloning. DNA amplification was carried out as described in Section 2.3.3. The p2494-KpnI-F and mTg-XbaI-R primers were used to amplify the carboxyl terminus of the Tg gene containing both p2494 and p2694 T-cell epitopes. The p2694-XbaI-R primer was designed to amplify 2 additional a.a. (-DA-) at the 3'-end of the p2694 T-cell epitope due to the differences between the two published mTg sequences[153;154]. The amplified PCR products were subject to electrophoresis on 4% (p2494 and p2694) and 1.5% (mTg-C) agarose gels, and the 72 bp (p2494), 81 bp (p2694), and 780 bp (mTg-C) fragments were purified as described in Section 2.3.3. Fragments were then digested with *KpnI* and *XbaI* and subcloned into a similarly digested and purified pcDNA3.1zeo$^+$ mammalian expression vector as described in Section 2.3.3.

2.6 AMPLIFICATION OF THE MUR INE IL-2 and IL-4 cDNA BY PCR AND SUBCLONING INTO pcDNA3.1zeo$^+$

The pGCVII mammalian expression vector (Eppendorf – 5 prime, Boulder, CO, USA) containing the murine IL-4 cDNA (423 bp) was a kind gift from Dr. Brian Barber (University of Toronto). Unfortunately, using restriction enzyme analysis, we were unable to determine where the gene was inserted; therefore we amplified the murine IL-4 cDNA insert from the vector using the polymerase chain reaction (PCR) and murine IL-4 specific primers. Similarly, we received the pcDV1 vector containing the mIL-2 cDNA (510 bp) from ATCC (Rockville, MD, USA). The murine IL-2 cDNA insert was also

amplified from the vector using the polymerase chain reaction and murine IL-2 specific primers due to the difficulties in obtaining sufficient amounts of the pcDV1-mIL-2 plasmid for future mIL-2 insert excision and ligation. Sequences of the oligonucleotide primers (5' → 3') were as follows: mIL-4-KpnI-F = GCG GTA CC*A* ***TG***G GTC TCA ACC CC, mIL-4-XbaI-R = CGT CTA GA*C* ***TA***C GAG TAA TCC ATT TGC, mIL-2-KpnI-F = CGG GTA CC*A* ***TG***T ACA GCA TGC AG and mIL-2-XbaI-R = CCT CTA GA*T* ***TA***T TGA GGG CTT GTT G (GibcoBRL, Burlington, ON, Canada). These primers included the native translation start and stop codons (bold and *italicized*) as well as unique *KpnI* and *XbaI* restriction sites (underlined) outside of the 5' and 3' coding regions (bold) respectively, to facilitate subcloning. DNA amplification was carried out as described in Section 2.3.3. The amplified PCR products were subject to electrophoresis on a 1.5 % agarose gel, and the 435 bp (mIL-4) and 522 bp (mIL-2) fragments were purified as described in Section 2.3.3. These fragments were then digested with *KpnI* and *XbaI* (Promega, Madison, WI, USA) and subcloned into a similarly digested and purified pcDNA3.1zeo$^+$ mammalian expression vector as described in Section 2.3.3.

2.7 PLASMID PREPARATIONS

2.7.1 Preparation of Fresh Competent *E. coli* using Calcium Chloride

Competent *Escherichia coli* (strain: XL1-Blue) cells were prepared using a modified calcium chloride protocol described in Current Protocols in Molecular Biology[155]. Briefly, 5 ml of Luria Bertani (LB) media (10 g tryptone peptone, 5 g yeast extract, and 10 g NaCl per liter) was inoculated with a single colony taken from a freshly

streaked LB agar plate, and incubated overnight at 37°C with vigorous shaking (~250 rpm). This starter culture was then diluted (1/100) into 100 ml of LB medium and incubated at 37°C with vigorous shaking (~250 rpm) until the culture OD_{600nm} was between 0.3-0.4 ($\leq 10^8$ cells/ml). The bacterial culture was aliquoted into two 50 ml polypropylene tubes (50 ml/tube) and incubated on ice for 5-10 minutes. Bacterial cells were harvested by centrifugation at 1600 x g for 7 min at 4°C. Bacterial pellets were resuspended in 10 ml of filter-sterilized ice-cold 0.1 M $CaCl_2$ and re-centrifuged at 1100 x g for 5 minutes at 4°C. Supernatant was discarded, and bacterial pellets were resuspended again in 10 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. Following another centrifugation (1100 x g for 5 minutes at 4°C), supernatant was removed by aspiration and each pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ containing 15% glycerol. Competent cells were then aliquoted into pre-chilled sterile microcentrifuge tubes (100 µl/tube) and stored at –70°C (maximum 6 months) for future transformations.

2.7.2 Transformation of Competent *E. coli* with Plasmid DNA or Ligation Reactions

Competent *Escherichia coli* (XL1-Blue) cells were transformed with various plasmid DNA or ligation reactions using a modified transformation protocol[46]. Briefly, competent cells were thawed on ice, gently mixed, and transferred to a pre-chilled microcentrifuge tube (100 µl). Plasmid DNA (1-50 ng) or 5 µl of a ligation reaction was then added to the cells and incubated on ice for 10-30 minutes. No DNA was added for a negative control. Next, the cells were heat-shocked for 45-50 seconds in a 42°C water bath, and then immediately returned on ice for 2 minutes. Cold LB media (900 µl) was

then added to each transformation reaction followed by a 60 minute incubation at 37°C with vigorous shaking (250 rpm). An aliquot of each transformation reaction (100 µl) was plated onto LB agar plates containing 100 µg/ml of ampicillin (Sigma Chemicals, St. Louis, MO, USA) and incubated overnight (12-16 hours) at 37°C.

2.7.3 Plasmid Purification

All plasmids were initially purified using Qiagen plasmid mini-prep kits (Mississauga, ON, Canada) to ensure correct plasmid sizes through restriction enzyme analysis (Section 2.7.4), test gene functionality (Section 2.9), and sequence cDNA inserts (Section 2.8). Plasmids used for DNA immunizations were purified using QIAGEN EndoFree™ Plasmid Giga Kits (Mississauga, ON, Canada). Briefly, 5 ml of LB media containing 100 µg/ml of ampicillin (Sigma Chemicals, St. Louis, MO, USA) was inoculated with a single isolated transformed XL1-Blue (*Escherichia coli*) colony taken from a freshly streaked selective plate, and incubated for ~8 hours at 37°C with vigorous shaking (~300 rpm). This starter culture was then diluted (1/1000) into 2.5 L of selective LB medium and incubated overnight at 37°C with vigorous shaking (~300 rpm). Bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C, and the bacterial pellet was resuspended in 125 ml of buffer P1 containing 100 µg/ml RNase A provided with the QIAGEN kit. An equivalent amount of buffer P2 was then added, mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for exactly 5 minutes to lyse the bacterial cells. Buffer P3 was immediately added following cell lysis, gently mixed to enhance precipitation of proteins, genomic DNA, and detergent, and poured into the QIAfilter Mega-Giga Cartridge. Following a 10

minutes incubation period, the bacterial cell lysate was obtained by vacuum filtration and washing with buffer FWB. Next, buffer ER was added to the filtered lysate and incubated on ice for 30 minutes. Buffer ER, or endotoxin removal buffer prevents lipopolysaccharide (LPS) molecules (cell membrane components of Gram negative bacteria) from binding to the resin in the QIAGEN-tips allowing purification of DNA containing less than 0.1 endotoxin units per μg plasmid DNA. Treated filtered lysate was then added to an equilibrated QIAGEN-tip 10 000 and allowed to empty by gravity flow. After the QIAGEN-tip was washed 2x with 300 ml of buffer QC, DNA was eluted using QN buffer. Plasmid DNA was then precipitated following the addition of 52.5 ml of isopropanol and then immediately centrifuged at ≥ 15 000 x g for 30 minutes at 4°C. The DNA pellet was washed with 10 ml of 70% endotoxin-free ethanol, recentrifuged (15 000 x g, 15 min, 4°C), air dried (~15 minutes), and resuspended in ~2 ml of 0.9% NaCl solution (pyrogen free). The double-stranded plasmid DNA absorbance was then read using a spectrophotometer at an optical density of 260 and 280 nm to determine DNA yield and purity. The A_{260}/A_{280} ratio for each plasmid prep were between 1.8 –1.9, with a characteristic yield of approximately 10 mg per column.

2.7.4 Restriction Enzyme Analysis of Plasmid DNA

Purified plasmid DNA was subject to restriction enzyme analysis to ensure the correct plasmid size and confirm the presence of the gene insert. All restriction enzymes were purchased from Promega (Madison, WI, USA) and each digestion was performed according to Promega's Product Information sheet provided with each restriction enzyme purchase. Briefly, 0.5 μg of supercoiled plasmid DNA was added to a solution containing

1X of the appropriate restriction enzyme buffer, 5 μg of acetylated BSA, and 5 units of the appropriate restriction enzyme (final volume = 20 μl), and then incubated at 37°C for 1 hour. If two restriction enzymes were required to release the gene insert from the plasmid, then 5 units of each restriction enzyme was added to the digestion reaction. The digested plasmids were then subjected to electrophoresis on agarose gel containing 0.5 μg/ml of ethidium bromide (Sigma Chemicals, St. Louis, MO, USA). Gel images were captured using the ChemImager™ 4000 (Canabera Packard Canada Ltd, Montreal, QU, Canada).

2.8 SEQUENCING OF CLONED cDNA

Selected plasmids were sent to the University of Toronto DNA Sequencing Facility for analysis. The full coding sequence and portions of the 5' and 3' flanking ends of the human and murine TSHR, mIL-4, mIL-2 cDNA inserts and p2494, p2694, and mTg-C cDNA segments were derived using the dideoxynucleotide chain termination method and AmpliTaq DNA polymerase (PE Biosystems). Custom primers for the hTSHR and mTSHR were designed based on the published sequences of Nagayama, et al.[25] and Stein, et al.[63] respectively (Table 2.1) and synthesized by GibcoBRL (Burlington, ON, Canada). Primers designed from the T7 promoter priming site and BGH reverse priming site of pcDNA3.1zeo$^+$ expression vector were also used to sequence the flanking ends of the human and murine TSHR cDNA inserts, as well as sequence the entire mIL-4, mIL-2, p2494, p2694, and mTg-C cDNA inserts.

TABLE 2.1 List of forward and reverse primers used to sequence cloned cDNA inserts

Primer Name	Primer Sequence (5' → 3')	Primer Coordinates[a]
hTSHR394R	GTA GAA GGA GTG TGA	374-394
hTSHR633F	TTC CAG	633-652
hTSHR1065F	AAA CCT TGA CAC TGA	1065-1084
hTSHR2052F	AGC TG	2052-2071
hTSHR2077R	TCC ACC AGG AAT ATG	2057-2077
	AAG AG	
	AGC CTC TCA TCA CTG	
	TTA GC	
	GGA GTT GCT AAC AGT	
	GAT GAG	
mTSHR174F	GAG GAC GAC TTC AGA	174-193
mTSHR344R	GTC AC	325-344
mTSHR946F	CTG CAG AGT TGC ATC	946-965
mTSHR1171R	TAT AG	1152-1171
mTSHR1377F	AAA TCA GGG GAA TCC	1377-1396
mTSHR2130F	TGG AG	2130-2150
mTSHR2298R	AAA CCA ACG ACC TCA	2279-2298
	TCC TC	
T7-F	GTC CTG CTC ATT CTG	863-882
BGH-R	CTA AC	1022-1039
	CAG AGG GAC GTG TTC	
	ATC CTG	
	GCA GTT CAT AGG TAT	
	CTT GC	
	TAA TAC GAC TCA CTA	
	TAG GG	
	TAG AAG GCA CAG TCG	
	AGG	

[a]Primer coordinates based on the hTSHR and mTSHR published sequences of Nagayama, et al.[25] and Stein, et al.[63] respectively and the pcDNA3.1zeo$^+$ vector sequence (InVitrogen, Carlsbad, CA, US).

2.9 EXPRESSION AND FUNCTIONALITY TESTS OF CLONED cDNA

Expression and functionality of cloned cDNA inserts were tested by transiently transfecting the Chinese hamster ovarian cell line (CHO-K1), or antigen presenting cell lines (TA3 or LS102.9) with pcDNA3.1zeo$^+$ containing the cloned insert of interest using TransFast™ Transfection Reagent (Promega, Madison, WI, USA). Transfection efficiency of each cell line was predetermined using the luciferase assay (Section 2.9.2). Transiently transfected cells or cell supernatants were then used in various assays to test cDNA insert expression and functionality.

2.9.1 Transient Transfection of CHO-K1, TA3, or LS102.9 Cells

CHO-K1 cells were plated in 24-well (1 x 10^5 cells/well) or 6-well plates (5 x 10^5 cells/well) one day prior to transfection so that the cells were ~70-80% confluent on the day of transfection. TA3 and LS 102.9 cells were plated in 6-well plates in serum-free media (0.5 ml of 2 x 10^6 cells/ml) the same day as the transfection. A 1:1 TransFast™ Reagent to DNA charge ratio was prepared in serum-free medium and incubated at room temperature for 15 minutes. Following careful removal of the growth medium, 1 ml of the TransFast™ Reagent/DNA mixture (a total of 0.5μg or 2.5 μg of DNA/well) was added to the CHO-K1 cells and incubated for 1 hour at 37°C. TA3 and LS102.9 cells were transfected with 0.5 ml of the TransFast™ Reagent/DNA mixture (a total of 3 μg of DNA/well) and also incubated for 1 hour at 37°C. At the end of the incubation period, 5 ml of prewarmed complete medium was added to each well and the plate was returned to the incubator for an additional 48 hours.

2.9.2 Luciferase Assay

Prior to transfecting CHO-K1, TA3, and LS 102.9 cell lines with pcDNA3.1zeo$^+$ containing the cloned cDNA of interest, transfection efficiency of each cell line was predetermined using a luciferase assay system. Each cell line was transiently transfected (Section 2.9.1) with varying amounts of either pGL3-Basic or pGL3-Control vectors (Promega, Madison, WI, USA) kindly provided by Dr. G. Paterno (Memorial University of Newfoundland). The pGL3 reporter vectors contain a modified firefly luciferase gene, which encodes a monomeric 61 kDa protein that catalyzes the oxidation of beetle luciferin with concomitant production of photons (light), which can be measured using a luminometer[46]. Unlike pGL3-Control plasmid, pGL3-Basic lacks eukaryotic promoter and enhancer sequences and therefore is unable to express the luciferase gene in mammalian cells making this vector a suitable control. Following transfection, cells were harvested, washed 1x with PBS, and then lysed with cell culture lysis reagent provided with the luciferase assay system (Promega, Madison, WI, USA). Twenty microlitres of cell lysate was then dispensed into Falcon tubes and the reaction initiated by pipetting 100 µl of luciferase assay reagent (substrate) into the tube. Light intensity was measured using a Monolight® 2010 luminometer and results are expressed in relative light units.

2.9.3 cAMP Assay: Testing Expression and Functionality of cloned hTSHR and mTSHR cDNA

The Biotrak™ cAMP competitive enzyme immunoassay (EIA) system from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden) was used to test the expression and functional activity of human and murine TSHR. CHO-K1 cells transiently transfected with either pcDNA3.1zeo$^+$ (control), pcDNA3.1zeo$^+$/hTSHR or pcDNA3.1zeo$^+$/mTSHR

were harvested following the 48 hour transfection period, washed, and resuspended in Ham's F12 medium supplemented with 0.1 % (w/v) BSA and 0.2 mg/ml 3-isobutyl-1-methylxanthine (Sigma Chemicals, St. Louis, MO, USA). CHO stably expressing native hTSHR (JP09 cells) that exhibit an increase of cAMP following stimulation with TSH[30] were used as a positive control. In 96-well plates, transiently transfected CHO-K1 cells or JP09 cells (4 x 10^4 cells/well) were incubated in the presence or absence of 5 mU/ml of bovine TSH (Sigma Chemicals, St. Louis, MO, USA) for 2 hours at 37°C. Immediately following stimulation, cells were lysed by the addition of lysis reagent 1A (2.5% dodecyltrimethylammonium bromide in .05 M acetate buffer, pH 5.8 containing .02% BSA and .01% preservative) provided with the Biotrak cAMP competitive EIA kit. Total cellular cAMP released from the lysed cells was measured using the reagents provided with the assay kit. Briefly, 100 µl of diluted cell lysate (1:10 to 1:40 in lysis reagent 1B) or standard concentrations of cAMP and 100 µl of rabbit anti-cAMP antibody were added to the wells of a 96-well plate pre-coated with anti-rabbit IgG and incubated at 4°C for 2 hours. cAMP conjugated to horseradish peroxidase was then added to each well (50 µl/well) and incubated for an additional hour at 4°C. After the plate was washed 4x with buffer, 150 µl of the enzyme substrate was dispensed into all wells and incubated at room temperature for 1 hour with moderate shaking on a plate shaker (~250 rpm). The reaction was terminated following the addition of 1 M sulfuric acid (100 µl/well) and then the plate was read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Duplicate samples were assayed in all experiments; results are expressed as pmol cAMP/ml.

2.9.4 CTLL-2 and CT.4S Bioassays: Testing Functionality of cloned mIL-2 and mIL-4 cDNA

The IL-2 and IL-4 dependent cell lines (CTLL-2 and CT.4S respectively) were used to test the supernatants of pcDNA3.1zeo$^+$/mIL-2 and pcDNA3.1zeo$^+$/mIL-4 transfected CHO-K1 cells for functional IL-2 and IL-4 expression. Supernatants from pcDNA3.1zeo$^+$ transfected CHO-K1 cells were used as a control. Sample supernatants were serially diluted using complete medium in a 96-well microwell plate (100 µl/well). CTLL-2 and CT.4S cells were harvested in active log-phase growth, washed 2x in complete medium, and resuspended at 1 x 10^5 cells/ml. Cells were then added to the wells containing the samples (100 µl/well), and incubated for 24 (CTLL-2) or 48 hours (CT.4S) at 37°C. Eight (CTLL-2) or 24 hours (CT.4S) prior to harvesting, ^{3}[H] thymidine (NEN Life Science Products, Boston, MA, USA) was added to each well (1 µCi/well) in 25 µl of culture medium. Cells were harvested using the Harvester 96® Mach III M (Tomtec, Hamden, CT, USA) and incorporated radioactivity was measured using the TopCount NXT™ Microplate Scintillation & Luminescence Counter (Canberra Packard Canada, Mississauga, Ontario, Canada).

2.9.5 T Cell Activation Assay: Testing the ability of transfected APC to activate peptide-specific T cell hybridomas

The APC lines, TA3 or LS 102.9, were transiently transfected with pcDNA3.1zeo$^+$ constructs containing mini-cDNA inserts encoding the pathogenic T cell epitopes p2494 or p2694 (Section 2.6). These transfected APC were used to activate the MHC class II-restricted T-cell hybridomas, 4A2, 4A12, and 5E8 recognizing p2494 and 6E10 recognizing p2694 (Section 2.2) peptides. In a flat-bottom 96-well plate, 10^5

hybridoma cells/well were incubated with an equal number of TA3 or LS 102.9 antigen presenting cells previously transfected with the indicated DNA constructs. Untransfected TA3 or LS 102.9 cells were used as controls. To ensure that the antigen presenting ability of the APCs remained intact following transfection, hybridomas were also incubated with the APCs previously transfected with pcDNA3.1zeo$^+$ in the presence of appropriate peptides (10 μg/ml). The cells were cultured in a total of 200 μl culture medium per well. After 24 hours, 100 μl of supernatant was collected from each well, transferred to a new plate, and kept frozen at –70°C. The relative IL-2 content of culture supernatant from the T cell activation assay was determined by thawing the microtitre plates and performing a CTLL-2 proliferation bioassay (section 2.9.4).

2.10 DNA IMMUNIZATION

2.10.1 Antigenic challenge of animals

Plasmid DNA was delivered intradermally (i.d.) to the shaved posterior-dorsal skin of kentamine/xylazine-anesthetized (CDMV, Inc., Quebec, Canada) mice (BALB/cJ or AKR/J) using a 30-guage needle. Each mouse received a total of 10 or 50 μg of the appropriate plasmid DNA (in 0.9% NaCl) given in five 10 μl i.d. inoculations. Mice that were co-injected with two different plasmids received 50 μg of each plasmid given in five 10 μl injections. Mice were boosted with the same amount of DNA in the same manner at 3 and 6 weeks postpriming. Blood samples were obtained from the retro-ocular plexus 8, 10 and in some cases 12 weeks after the initial immunization. Mice were sacrificed at 10 or 12 weeks and thyroids were removed for gross and histological examination.

2.10.2 Detection of TSHR-specific Autoantibody by Flow Cytometry

Chinese hamster cells (CHO) stably transfected with human TSHR-ECD cDNA (GPI9-5 cells) or control vector-transfected CHO cells (CHO1) were used to detect the presence of TSHR-specific autoantibody in immune mouse sera. GPI9-5 or CHO1 cells were detached from the culture flasks after 2 washes with PBS using non-enzymatic cell dissociation solution (Sigma Chemicals, St. Louis, MO, USA), washed 2x and transferred into a 96-V shaped microwell plate (2 x 10^5 cells/well). Cells were centrifuged at 500 x g for 5 minutes at 4°C, washed once with PBS (1x) containing 1% (w/v) BSA (w/v) and 0.1% (w/v) NaN_3 (FACs buffer) and the supernatant removed by aspiration. Cells were then incubated on ice for 30 minutes at 4°C with 50 µl of FACs buffer containing 10 µl of neat mouse sera. Purified A10 mAb (IgG2b) known to recognize TSHR-ECD amino acids 22-35[147] was used as a positive control (10 µg/ml) and purified HB65 mAb (IgG2a) specific for nucleoprotein of the influenza type A virus was used as a nonspecific negative control (10 µg/ml). Cells were washed 4x with 150 µl of FACs buffer and centrifuged as described above. Cells were subsequently incubated at 4°C in the dark for 30 minutes with 50 µl of flourescein-conjugated γ-chain and F_c-specific goat anti-mouse IgG (Sigma Chemicals, St. Louis, MO, USA) diluted (1:80) in FACs buffer. Cells were washed 4 more times with FACs buffer, suspended in 500 µl of 1x PBS buffer containing 1% (w/v) paraformaldehye (Sigma Chemicals, St. Louis, MO, USA) and transferred to "Falcon" tubes. The fluorescence of 10 000 cells/tube was analyzed by a FACStar Plus flow cytometer and Cellquest software (Becton-Dickinson Inc., Franklin Lakes, NJ,

USA). Results are expressed as the mean channel fluorescence (MCF) ratio between GPI9-5 and CHO1.

2.10.3 Detection of Thyroid-Stimulating Autoantibodies to TSHR using cAMP Assay

Thyroid-stimulating autoantibodies were detected in mouse sera using CHO cells stably expressing native hTSHR (JP09)[30]. Briefly, 4 x 10^4 JP09 cells were plated in 96-well flat-bottomed plates and cultured for 24 hours in growth medium. Medium in the wells was removed by aspiration and immune mouse sera diluted (1:10) in Ham's F12 containing 0.1 % (w/v) BSA, 0.2 mg/ml 3-isobutyl-1-methylxanthine (Sigma Chemicals, St. Louis, MO, USA) was added to these wells (90 µl/well) and incubated for 2 hours at 37°C. Immediately following stimulation, cells were lysed by the addition of 10 µl of lysis reagent 1A (2.5% dodecyltrimethylammonium bromide in assay buffer) provided with the Biotrak cAMP competitive enzyme immunoassay kit. Cell lysates were diluted with lysis reagent 1B (1:10) and total cellular cAMP released from the cells was measured according to the manufacturer's protocol and as previously described in Section 2.9.3. Duplicate samples were assayed in all experiments; results are expressed as pmol cAMP/ml.

2.10.4 Total T4 Determination

A radioimmunoassay (RIA) kit (DYNOtest; BRAHMS Diagnostica GmbH, Berlin, Germany) was used for total T4 determination in which mouse T4 competes with ^{125}I-T4 for the antigen binding sites of a T4-specific antibody immobilized on the inner surface of plastic tubes. A standard curve was constructed with reference standards provided by the manufacturer; TT4 values are expressed in µg/dl.

2.10.5 Thyroid Histology

Thyroid glands were removed *en block* (still attached to the trachea) 10 or 12 weeks postpriming and fixed in 10% buffered formalin. The lobes were then dissected from the trachea and embedded in methacrylate. Approximately 21 sections at 3.0 μm intervals were obtained throughout each gland. They were fixed to glass slides and stained with hematoxylin and eosin. Thyroids were scored for the presence of mononuclear cell infiltration as previously described[156]: 0 = no infiltration; 1 = interstitial accummulation of inflammatory cells; 2 = one or more foci of inflammatory cells at least the size of one follicle; 3 = extensive infiltration, 10-40% of the total area, 4 = extensive infiltration, 40-80% of the total area; 5 = extensive infiltration, > 80% of the total area. The highest infiltration score observed per gland was assigned to each mouse.

2.11 PEPTIDES

2.11.1 Peptide Synthesis

Peptides designated hTSHR438-449 [ILLTSHYKLNVP], and hTSHR716-731 [STDIQVQKVTHDMRQG] were synthesized to >80 % purity at Sigma-Genosys Ltd (The Woodlands, TX, USA). The Tg peptides p2494 (a.a. 2494-2510) [GLINRAKAVK QFEESQG] and p2694 (a.a. 2694-2711) [C(acm)SFWSKYIQTLKDADGAK] and mouse lysozyme peptide [DRGDQSTDYFILFINSR] were synthesized to >80 % purity at the Alberta Peptide Institute (Edmonton, Alberta, Canada). All peptides were blocked with an acetyl group at the N-terminal and an amide group at the C-terminal end. Mass spectrometry and HPLC analysis were performed on each peptide to verify composition

and purity. Human TSHR peptides used to immunize mice were chosen using overlapping A and B motifs from Altuvia et al.[157], as outlined in Section 2.11.2.

2.11.2 Algorithm-Based Search for A^k-Binding Peptides in the Human TSHR

From the study of Altuvia et al.[157], two motifs were used to screen hTSHR for peptides that may bind to the A^k molecule (Table 2.2). The Pôle Bio-Informatique Lyonnais: Network Protein Sequence analysis website (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattern.html) was used to scan the 764 amino acid residues of the human TSHR molecule. Using this program, sequences were found within the human TSHR that fit both motif A and motif B. We chose peptides in which motif A and motif B completely overlapped within the human peptide sequences.

2.11.3 Peptide Immunizations

AKR/J (H-2^k) mice were immunized with 200 nmol of hTSHR438-449 or hTSHR716-731 peptides in a 1:1 emulsion in Complete Freund's Adjuvant (CFA) (with *Mycobacterium butyricum*, Difco Laboratories Inc., Detroit, MI, USA). Each mouse was immunized subcutaneously (under ether anaesthesia) at 2 sites (base of tail and interscapular region).

2.11.4 Proliferation Assay: Induction of Antigen-specific LNCs

Nine days following peptide immunization, mice were euthanised with ether and their draining inguinal, axillary, and brachial lymph nodes were collected aseptically. A single cell suspension was prepared by passing the lymph node tissue through a sterile stainless steel wire mesh (Sigma, St. Louis, MO, USA), and washed in complete DMEM. LNC were cultured in 96-well plates at a concentration of 4×10^5 cells/well for 4 days in

Table 2.2 Motifs A and B within I-A^k-binding peptides[157]

Position #	Amino Acid Attributes	[Includes]/{Excludes} A.A.
MOTIF A		
1	Hydrogen acceptor, polar, not hydrophobic, not small, not aliphatic	[DEHNQ]
2	Not an amide	{NQ}
3	Any amino acid	any amino acid
4	Medium-sized, aliphatic, hydrophobic, no charge, not aromatic or an amide	[ILTV]
5	Any amino acid	any amino acid
6	Not aromatic	{FHWY}
7	Hydrophobic, no charge, not an amide	[ACFILMPTVWY]
[DEHNQ]-{NQ}-X-[ILTV]-X-{FHWY}-[ACFILMPTVWY]		
MOTIF B		
1	Hydrogen acceptor, not small, polar, not aliphatic	[CDEHNQY]
2	Not negatively charged	{DE}
3	Any amino acid	any amino acid
4	Medium-sized, aliphatic, hydrophobic, no charge, not aromatic or an amide	[ILTV]
5	Hydrogen acceptor, non-hydrophobic, not small, not aliphatic	[DEHQN]
[CDEHNQY]-{DE}-X-[ILTV]-[DEHQN]		

the presence of titrated amounts of the appropriate antigen in 200 μl microcultures. Eighteen hours prior to harvesting, 1 μCi of ^{3}[H] thymidine was added to each culture in 25 μl of culture medium. Cells were harvested and incorporated radioactivity was measured as described in Section 2.9.4. Stimulation index is defined as CPM in the presence of antigen/CPM in the absence of antigen.

2.11.5 Cytokine ELISA for assessment of cytokines in culture

The concentration of interferon-γ, IL-4, IL-2, and IL-10 in 48 hour culture supernatants collected from primed LNCs cultured in the presence of 15 μM of indicated Ag were determined using a cytokine sandwich ELISA. Polyvinyl chloride (PVC) 96-well ELISA plates were coated with purified anti-cytokine capture mAbs (50 μl) overnight at 4°C diluted in a phosphate binding solution (0.1 M Na_2HPO_4, pH 9.0 with 0.1 M NaH_2PO_4) to a concentration of 1 μg/ml (anti-IL-2, IL-4, and IL-10) or 4 μg/ml (anti-IFN-γ). Anti-IL-2 capture mAb (JES6-1A12 antibody) was purchased from PharMingen (San Diego, CA, USA), while anti-IL-10 capture antibody was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Anti-IL-4 and anti-IFN-γ capture mAbs were purified and concentrated as described in Section 2.2 from the supernatant of the hybridoma cell lines 11B11 (HB188, IgG1, anti-IL-4)[158] and R4-6A2 (HB170, IgG1, anti-IFN-γ)[159] previously purchased from ATCC. Plates were washed 2x with PBS and blocked for 2 hours at room temperature with 1% (w/v) BSA. After washing 3x with PBS-Tween, 100 μl of sample supernatants, and serially diluted recombinant cytokines were added to the wells. Recombinant murine IL-2 and IFN-γ were obtained from

PharMingen (San Diego, CA, USA), while IL-4 and IL-10 cytokines were obtained from Peprotech Inc. (Rocky Hill, NJ, USA). The plates were incubated overnight at 4°C, washed 3x with PBS-T, and incubated with 100 µl of 1 µg/ml (anti-IL-4 and IFN-γ) or 2 µg/ml (anti IL-2 and IL-10) of biotinylated anti-cytokine mAb for 1 hour at room temperature. Biotinylated anti-IL-2 (JES6-5H4, rat IgG2b), IL-4 (BVD6-24G2, rat IgG1) and IFN-γ (XMG1.2, rat IgG1) detection antibodies were obtained from PharMingen (San Diego, CA, USA), while biotinylated anti-IL-10 antibody was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). The plates were washed 4x with PBS-T and then incubated with 100 µl of streptavidin-alkaline phosphatase (Sigma Chemicals, St. Louis, MO, USA) diluted (1:3000) in 1% BSA-PBS-T. After a one-hour incubation at room temperature and washing (5x with PBS-T), 100 µl of p-nitrophenylphosphate substrate (Sigma Chemicals, St. Louis, MO, USA) diluted to 1mg/ml in 10% diethanolamine (Fisher Scientific, Napean, Ontario, Canada) was added. Following a one-hour incubation period at room temperature, the plate was read (405 nm) using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.11.6 Antibody Blocking Assays: Determination of A^k or E^k peptide binding

To determine whether hTSHR peptides bind to I-A^k or I-E^k MHC class II molecules, primed LNCs (Section 2.11.3) were cultured in the presence of titrated amounts of the appropriate antigen and either anti-IA^k, anti-IE^k, or anti-influenza A nucleoprotein (non-specific control) mAbs (10 µg/ml) for 24 hours at 37°C. MAbs were purified and concentrated as described in Section 2.2 from culture supernatants of the hybridoma cell lines 10.3.62 (TIB 92, IgG2a, anti-IA^k)[160], 14-4-4S (HB32, IgG2a, anti-

IEk)[161], and H16-L10-4R5 (HB65, IgG2a, anti-influenza A nucleoprotein (NP))[162] obtained from ATCC. Six hours prior to harvesting, 1 μCi of ^{3}[H]-thymidine was added to each culture well in 25 μl of medium. Cells were harvested and incorporated radioactivity was measured as described in Section 2.9.4. Percent inhibition of proliferation by blocking mAb is expressed as (1-(cpm in the presence of mAb)/(cpm in the absence of mAb)) x 100.

2.11.7 EAT Induction

To determine whether the hTSHR peptides were thyroiditogenic, mice (n=6) were boosted 3 weeks after initial priming (Section 2.11.3) with 100 nmol of peptide in Incomplete Freund's Adjuvant (IFA). After two weeks, these mice were bled, and their sera were tested in an ELISA (Section 2.11.8). Their thyroids were also removed for histological analysis (Section 2.10.5).

2.11.8 Detection of peptide-specific Ab by ELISA

An indirect antibody ELISA was used to detect the presence of hTSHR peptide-specific IgG in pooled mice sera (5-6 mice/group). PVC microwell plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with the immunizing peptide or non-specific lysozyme peptide (0.2 μg/well) dissolved in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were then blocked overnight at 4°C with PBS containing 1% BSA. Serially diluted pooled sera samples were added to the wells (100 μl/well) for one hour at room temperature and then washed three times with PBS-T. The second antibody, an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA) diluted (1:3000) in 1% BSA-PBS-Tween was then added to each well

(100 µl/well). After an hour incubation at room temperature, the plates were washed again 3x with PBS-T followed by the addition of 100 µl of p-nitrophenylphosphate substrate (Sigma, St. Louis, MO, USA) solution (1 mg/ml p-nitrophenylphosphate in 10% diethanolamine, pH 9.8). Absorbance of the p-nitrophenylphosphate product was measured 30 minutes later at 405 nm using an automated microplate reader (Molecular Devices, Sunnydale, CA, USA).

CHAPTER THREE

INDUCTION OF HYPERTHYROIDISM IN BALB/cJ MICE BY GENETIC IMMUNIZATION WITH hTSHR

3.1 ABSTRACT

An animal model that exhibits most of the clinical signs and symptoms of GD would be a valuable tool for studying the pathology and aetiology of this autoimmune thyroid disease (AITD). Previous attempts to establish a mouse model of GD by i.m. immunization of mice with plasmid DNA encoding the hTSHR gene have yielded conflicting results. In this study, we have used DNA immunization to examine the effect of several parameters on the immune response including: a) homologous (murine; pcDNA3.1zeo$^+$/mTSHR) versus heterologous (human; pcDNA3.1zeo$^+$/hTSHR) TSHR Ag, b) Ag dose (50 μg vs. 10 μg of DNA), c) cytokine-vector co-delivery (pcDNA3.1zeo$^+$/IL-2 or pcDNA3.1zeo$^+$/IL-4), and d) genetic background of the host (BALB/cJ versus AKR/J mice). Disease induction was assessed by measuring TSHR-specific antibodies, TSAb activity, and TT4 levels in the sera of immunized mice as well as by histological examination of the thyroid gland. Surprisingly, very few BALB/cJ mice produced autoantibodies specific for the TSHR molecule following i.d. DNA immunization with pcDNA3.1zeo$^+$ encoding the homologous (murine) TSHR, regardless of antigen dose or co-delivery of cytokine-vectors indicating that tolerance to the mTSHR could not be broken. In contrast, between 50-60 % of BALB/cJ and AKR/J mice produced TSHR-specific Ab following i.d. immunization with the heterologous (human) TSHR cDNA. Three out of the 8 BALB/cJ mice immunized with the larger dose of hTSHR-encoding plasmid also became thyrotoxic with TT4 levels as high as 16.21 μg/dl

(normal range = 5.09-6.51 μg/dl), and thyroids from 2 of these 3 thyrotoxic mice exhibited signs of hyperactivity, but lacked lymphocytic infiltration. Thyroid hyperactivity, however, did not coincide with TSAb activity, which may have been masked by TSBAbs or neutral TSHR antibodies. Surprisingly, co-delivery of either mIL-2 or mIL-4 cytokine vectors did not enhance antibody and hyperthyroidism induction in BALB/cJ mice for unknown reasons. These results confirm the efficacy of i.d. DNA immunization in inducing immune responses against the encoded heterologous hTSHR as well as the ability of hTSHR autoantibodies to recognize and bind to mTSHR determinants stimulating thyroid hyperactivity and excessive hormone production.

3.2 INTRODUCTION

Currently, an animal model developing spontaneous GD does not exist; thus much of the research has focused on experimentally inducing GD in mice. Numerous attempts using purified TSHR from thyrocytes or recombinant protein expressed in bacteria or insect cells and conventional immunization protocols have failed to establish a disease model (Section 1.3.1 and 1.3.3). However, some new approaches for generating animal models of Graves' hyperthyroidism have recently been demonstrated. First, a novel immunization protocol described by Shimojo et al.[75] using transfected fibroblasts coexpressing hTSHR and MHC class II molecules induced hyperthyroidism in a small proportion (~ 20%) of immunized syngeneic AKR mice. This finding was later confirmed by two other groups[77;78]. Subsequently, using a variation of Shimojo's protocol, Kaithamana, et al.[80] were able to induce hyperthyroidism in almost 100% of the BALB/c mice receiving i.p. injections of B lymphoblastoid cells expressing TSHR. Although these studies reproduced several major features of GD, some of the drawbacks include cell line availability for only certain strains of mice, minimal lymphocytic thyroid infiltration, as well as the unexplained widespread immune activation recently reported by Yan et al.[79] Genetic immunization with an eukaryotic expression vector containing hTSHR cDNA has also proven to be useful in inducing hyperthyroidism in a small proportion (~ 20%) of outbred NMRI [84], but not inbred BALB/c mice[83]. However, the low rate of disease induction, as well as the inability of other research groups[85;163] to reproduce comparable results using similar immunization regimes, necessitates further investigation.

Since plasmid DNA can be manipulated to alter the quantitative and qualitative aspects of an immune response, it prompted us to modify previous DNA immunization protocols in an effort to establish a more reliable method for inducing GD in mice. First, in lieu of i.m. DNA immunization, mice were immunized intradermally with various doses of plasmid DNA because the skin, in contrast to muscle, has an established immune surveillance function carried out by a variety of professional antigen presenting cells including specialized bone-marrow derived Langerhans cells (LCs)[112;164;165]. Following direct transfection with plasmid DNA or internalization of antigen produced by transfected keratinocytes (skin cells), LCs migrate to the draining lymph nodes and present Ag to T and B cells[106]. Second, since GD is a Th2 mediated autoimmune disease[9;166], attempts to bias the immune response toward a Th2 type of response were made through co-delivery of plasmid DNA encoding mIL-4, a cytokine known to promote Th cell differentiation toward a Th2 pathway[167]. Third, although there is 86% homology between the human and murine TSHR sequences, limited a.a. differences may alter the tertiary structure of the receptor and cause production of TSHR-specific Abs that cannot bind or stimulate the endogenous mTSHR. The fact that only a minority of hTSHR antibodies that bind to the mTSHR act as agonist, stimulating the receptor, agrees with this interpretation[64-66]. To circumvent this, both the homologous (murine) and heterologous (human) TSHR cDNA were used as DNA immunogens. Finally, to determine whether the genetic background of the host plays a role in disease susceptibility, i.d. immunizations were performed in two different strains of mice (BALB/cJ and AKR/J).

3.3 RESULTS

3.3.1 Sequencing of cloned cDNA inserts

The dideoxynucleotide chain termination method was used to sequence the entire coding region and portions of the 5' and 3' flanking regions of human and murine TSHR cDNA and murine IL-4 and IL-2 cytokine inserts. The nucleotide and derived amino acid sequences of the cloned cDNA inserts are shown in Figures 3.1-3.3. The hTSHR sequence (Figure 3.1) was almost identical to the published sequence[25] with the exception of two nucleotide substitutions at positions 1477 (g → a) and 1801 (c → t). The latter nucleotide substitution caused an amino acid change at position 601 (H → Y). The sequence of mTSHR (Figure 3.2), mIL-4, and mIL-2 (Figure 3.3A & B respectively), on the other hand, were identical with the published sequences[63;168;169].

3.3.2 Restriction Enzyme Analysis of Plasmid DNA

Purified plasmid DNA was subject to various restriction enzyme digestions and gel electrophoresis to ensure correct plasmid size and confirm the presence of cDNA inserts (Figure 3.4). *KpnI* digested each plasmid at one site yielding the actual size of each plasmid: pcDNA3.1zeo$^+$ = 5015 bp (lane 1), pcDNA3.1zeo$^+$/hTSHR = 7428 bp (lane 2), pcDNA3.1zeo$^+$/mTSHR = 7249 (lane 3), pcDNA3.1zeo$^+$/mIL-2 = 5469 bp (lane 4), and pcDNA3.1zeo$^+$/mIL-4 = 5382 (lane 5). The hTSHR insert (~ 2.5 kb) was released when pcDNA3.1zeo$^+$/hTSHR was digested with *XhoI* (lane 6), while mIL-2 (~0.5 kb), mIL-4 (~0.4 kb), and mTSHR (~ 2.4 kb) were released when digested with both *KpnI* and *XbaI* (lane 7-9 respectively).

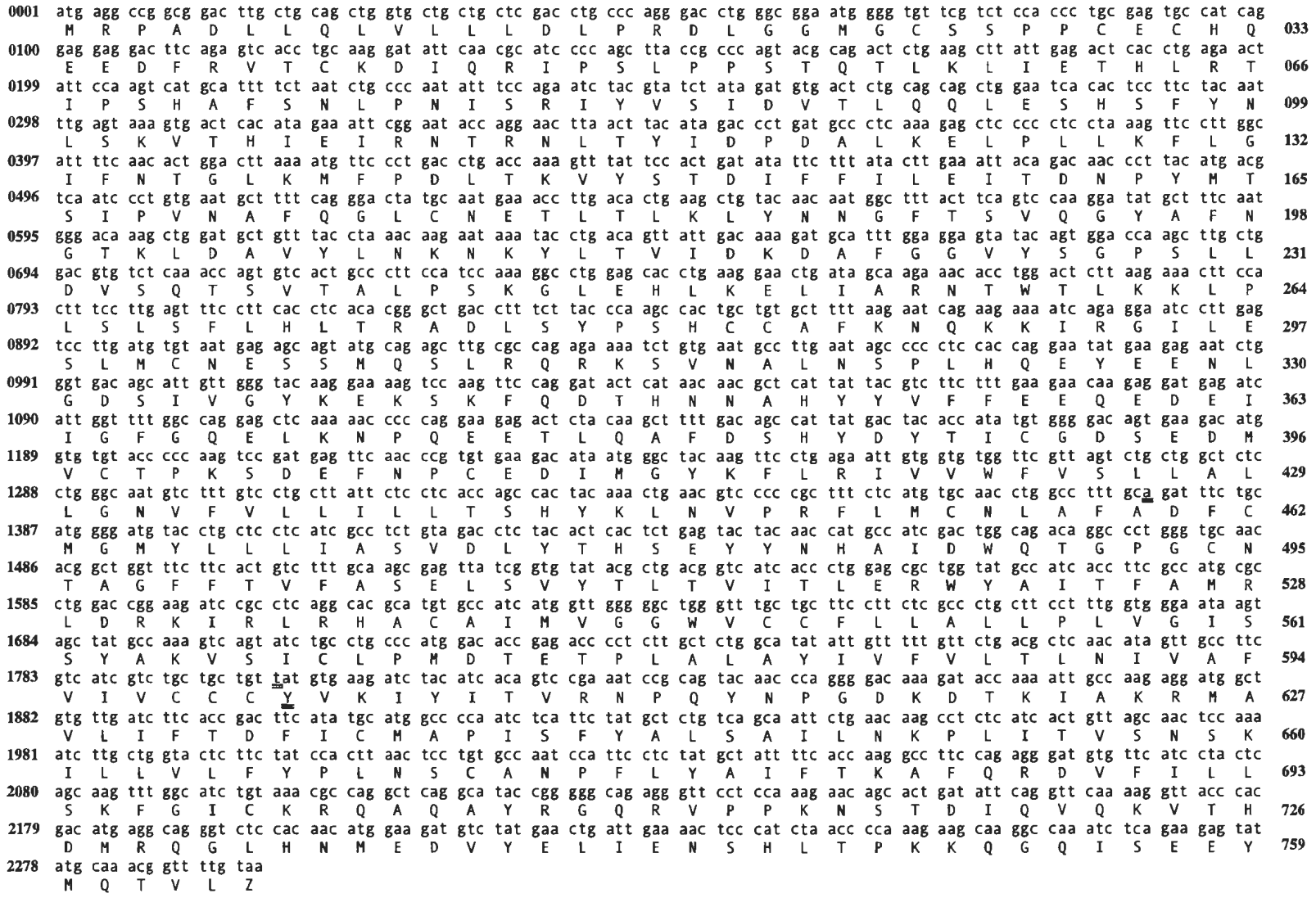


Figure 3.1 Nucleotide and derived amino acid sequence of the cDNA encoding the hTSHR protein. The sequence is identical to the published hTSHR sequence with the exception of 2 nucleotide substitutions at positions 1377 (g → a) and 1801 (c → t) (double underlined). The latter nucleotide sustitution caused an amino acid change at position 601 (H → Y) (double underlined).

```
0001 atg agg cca ggg tcc ctg ctg ctg ctt gtt ctg ctg ctc gcc ctg tcc agg agc ctg cgg ggc aaa gag tgt gcg tct cca ccc tgt gag tgt cac cag
     M  R  P  G  S  L  L  L  L  V  L  L  L  A  L  S  R  S  L  R  G  K  E  C  A  S  P  P  C  E  C  H  Q    033
0100 gag gac gac ttc aga gtc acc tgc aag gag ctc cac cga atc ccc agc ctg ccg ccc agc acc cag act ctg aag ctc atc gag act cat ctg aag acc
     E  D  D  F  R  V  T  C  K  E  L  H  R  I  P  S  L  P  P  S  T  Q  T  L  K  L  I  E  T  H  L  K  T    066
0199 ata ccc agt ctt gca ttt tcg agt ctg ccc aat att tcc agg atc tat tta tct ata gat gca act ctg cag cgg ctg gaa cca cat tct ttc tac aat
     I  P  S  L  A  F  S  S  L  P  N  I  S  R  I  Y  L  S  I  D  A  T  L  Q  R  L  E  P  H  S  F  Y  N    099
0298 ttg agt aaa atg act cac ata gaa atc cgg aac acc aga agc tta acc tat ata gac cct gat gcc ttg aca gag ctc ccc ttg ctc aag ttt ctt ggc
     L  S  K  M  T  H  I  E  I  R  N  T  R  S  L  T  Y  I  D  P  D  A  L  T  E  L  P  L  L  K  F  L  G    132
0397 att ttc aat act gga ctt aga ata ttc cct gac ttg acc aaa att tat tcc acg gac ata ttc ttt ata ctt gaa atc aca gac aac cct tac atg act
     I  F  N  T  G  L  R  I  F  P  D  L  T  K  I  Y  S  T  D  I  F  F  I  L  E  I  T  D  N  P  Y  M  T    165
0496 tcg gtc cct gaa aac gca ttc cag ggc cta tgc aat gaa acc ttg acc ctg aaa ctg tac aac aat gga ttt act tca gtc caa gga cat gct ttc aat
     S  V  P  E  N  A  F  Q  G  L  C  N  E  T  L  T  L  K  L  Y  N  N  G  F  T  S  V  Q  G  H  A  F  N    198
0595 gga aca aag ctg gat gct gtt tac cta aac aag aat aaa tac ctg aca gct ata gac aac gat gcc ttt gga gga gta tac agt gga cca act ttg cta
     G  T  K  L  D  A  V  Y  L  N  K  N  K  Y  L  T  A  I  D  N  D  A  F  G  G  V  Y  S  G  P  T  L  L    231
0694 gat gtg tct tcc acc agc gtc act gcc ctt cct tcc aaa ggc ctg gag cac ctc aaa gaa ctg atc gca aaa gac acc tgg act ctc aaa aag ctc ccg
     D  V  S  S  T  S  V  T  A  L  P  S  K  G  L  E  H  L  K  E  L  I  A  K  D  T  W  T  L  K  K  L  P    264
0793 ctg tcg ttg agt ttc ctc cac ctc act cgg gct gac ctc tct tac ccg agc cac tgc tgc gct ttt aag aac cag aag aaa atc agg gga atc ctg gag
     L  S  L  S  F  L  H  L  T  R  A  D  L  S  Y  P  S  H  C  C  A  F  K  N  Q  K  K  I  R  G  I  L  E    297
0892 tct ttg atg tgt aat gag agc agt atc cgg aac ctt cgt caa agg aaa tca gtg aac atc ttg agg ggt ccc atc tac cag gaa tat gaa gaa gat ccg
     S  L  M  C  N  E  S  S  I  R  N  L  R  Q  R  K  S  V  N  I  L  R  G  P  I  Y  Q  E  Y  E  E  D  P    330
0991 ggt gac aac agt gtt ggg tac aaa caa aac tcc aag ttc cag gag agc cca agc aac tct cac tat tac gtc ttc ttt gaa gaa caa gag gat gag gtc
     G  D  N  S  V  G  Y  K  Q  N  S  K  F  Q  E  S  P  S  N  S  H  Y  Y  V  F  F  E  E  Q  E  D  E  V    363
1090 gtt ggt ttc ggc caa gag ctc aaa aat cct cag gaa gag act ctc caa gcc ttc gag agc cac tat gac tac acg gtg tgt ggg gac aac gag gac atg
     V  G  F  G  Q  E  L  K  N  P  Q  E  E  T  L  Q  A  F  E  S  H  Y  D  Y  T  V  C  G  D  N  E  D  M    396
1189 gtg tgt acc ccc aag tcg gac gag ttt aac ccc tgt gaa gat atc atg ggc tac agg ttc ctg aga atc gtg gtg tgg ttt gtc agt ctg ctg gct ctc
     V  C  T  P  K  S  D  E  F  N  P  C  E  D  I  M  G  Y  R  F  L  R  I  V  V  W  F  V  S  L  L  A  L    429
1288 ctg ggc aat atc ttc gtc ctg ctc att ctg cta acc agc cac tac aaa ttg acc gtg ccg cgg ttc ctc atg tgc aac ttg gcc ttt gca gat ttc tgc
     L  G  N  I  F  V  L  L  I  L  L  T  S  H  Y  K  L  T  V  P  R  F  L  M  C  N  L  A  F  A  D  F  C    462
1387 atg ggg gta tac ctg ctt ctc att gcc tct gta gac ctg tac aca cac tct gag tac tac aac cac gcc atc gac tgg cag acg ggc cct ggg tgc aac
     M  G  V  Y  L  L  L  I  A  S  V  D  L  Y  T  H  S  E  Y  Y  N  H  A  I  D  W  Q  T  G  P  G  C  N    495
1486 acg gct ggc ttc ttc act gtt ttc gcc agt gag tta tca gtg tac aca ctg acg gtc atc acc ctg gag cga tgg tac gcc atc acc ttc gcc atg cgc
     T  A  G  F  F  T  V  F  A  S  E  L  S  V  Y  T  L  T  V  I  T  L  E  R  W  Y  A  I  T  F  A  M  R    528
1585 ctg gat agg aag atc cgc ctc agg cac gcg tac acc atc atg gct ggg ggc tgg gtt tcc tgc ttc ctt ctc gcc ctg ctc ccg atg gtg gga atc agc
     L  D  R  K  I  R  L  R  H  A  Y  T  I  M  A  G  G  W  V  S  C  F  L  L  A  L  L  P  M  V  G  I  S    561
1684 agc tat gcc aag gtc agc atc tgc ctg cca atg gac acc gac acc cct ctt gca ctc gca tac att gtc ctc gtt ctg ctg ctc aat gtt gtt gcc ttt
     S  Y  A  K  V  S  I  C  L  P  M  D  T  D  T  P  L  A  L  A  Y  I  V  L  V  L  L  L  N  V  V  A  F    594
1783 gtt gtc gtc tgt tcc tgc tat gtg aag atc tac atc acg gtc cga aat ccc cag tac aac cct cga gat aaa gac acc aag att gcc aag agg atg gct
     V  V  V  C  S  C  Y  V  K  I  Y  I  T  V  R  N  P  Q  Y  N  P  R  D  K  D  T  K  I  A  K  R  M  A    627
1882 gtg ttg atc ttc act gac ttc atg tgc atg gcg ccc atc tcc ttc tat gcg ctg tcg gca ctt atg aac aag cct cta atc act gtt act aac tcc aaa
     V  L  I  F  T  D  F  M  C  M  A  P  I  S  F  Y  A  L  S  A  L  M  N  K  P  L  I  T  V  T  N  S  K    660
1981 atc ttg ttg gtt ctc ttc tac ccc ctc aac tcc tgt gcc aat ccg ttt ctc tat gct att ttc acc aag gcc ttc cag agg gac gtg ttc atc ctg ctc
     I  L  L  V  L  F  Y  P  L  N  S  C  A  N  P  F  L  Y  A  I  F  T  K  A  F  Q  R  D  V  F  I  L  L    693
2080 agc aag ttt ggc atc tgc aaa cgc cag gcc cag gcc tat cag ggt cag aga gtc tgt ccc aac aat agc act ggt att cag atc caa aag att ccc cag
     S  K  F  G  I  C  K  R  Q  A  Q  A  Y  Q  G  Q  R  V  C  P  N  N  S  T  G  I  Q  I  Q  K  I  P  Q    726
2179 gac acg agg cag agt ctc ccc aac atg caa gat acc tat gaa ctg ctt gga aac tcc cag cta gct cca aaa ctg cag gga caa atc tca gaa gag tat
     D  T  R  Q  S  L  P  N  M  Q  D  T  Y  E  L  L  G  N  S  Q  L  A  P  K  L  Q  G  Q  I  S  E  E  Y    759
2278 aag caa aca gcc ttg taa
     K  Q  T  A  L  Z
```

Figure 3.2 Nucleotide and derived amino acid sequence of the cDNA encoding the mTSHR protein. The sequence is completely identical to the published mTSHR sequence.

A

```
001  atg ggt ctc aac ccc cag cta gtt gtc atc ctg ctc ttc ttt ctc gaa tgt acc agg agc
       M   G   L   N   P   Q   L   V   V   I   L   L   F   F   L   E   C   T   R   S   020
061  cat atc cac gga tgc gac aaa aat cac ttg aga gag atc atc ggc att ttg aac gag gtc
       H   I   H   G   C   D   K   N   H   L   R   E   I   I   G   I   L   N   E   V   040
121  aca gga gaa ggg acg cca tgc acg gag atg gat gtg cca aac gtc ctc aca gca acg aag
       T   G   E   G   T   P   C   T   E   M   D   V   P   N   V   L   T   A   T   K   060
181  aac acc aca gag agt gag ctc gtc tgt agg gct tcc aag gtg ctt cgc ata ttt tat tta
       N   T   T   E   S   E   L   V   C   R   A   S   K   V   L   R   I   F   Y   L   080
241  aaa cat ggg aaa act cca tgc ttg aag aag aac tct agt gtt ctc atg gag ctg cag aga
       K   H   G   K   T   P   C   L   K   K   N   S   S   V   L   M   E   L   Q   R   100
301  ctc ttt cgg gct ttt cga tgc ctg gat tca tcg ata agc tgc acc atg aat gag tcc aag
       L   F   R   A   F   R   C   L   D   S   S   I   S   C   T   M   N   E   S   K   120
361  tcc aca tca ctg aaa gac ttc ctg gaa agc cta aag agc atc atg caa atg gat tac tcg
       S   T   S   L   K   D   F   L   E   S   L   K   S   I   M   Q   M   D   Y   S   140
421  tag
       Z
```

B

```
001  atg tac agc atg cag ctc gca tcc tgt gtc aca ttg aca ctt gtg ctc ctt gtc aac agc
       M   Y   S   M   Q   L   A   S   C   V   T   L   T   L   V   L   L   V   N   S   020
061  gca ccc act tca agc tcc act tca agc tct aca gcg gaa gca cag cag cag cag cag cag
       A   P   T   S   S   S   T   S   S   S   T   A   E   A   Q   Q   Q   Q   Q   Q   040
121  cag cag cag cag cag cag cac ctg gag cag ctg ttg atg gac cta cag gag ctc ctg agc
       Q   Q   Q   Q   Q   Q   H   L   E   Q   L   L   M   D   L   Q   E   L   L   S   060
181  agg atg gag aat tac agg aac ctg aaa ctc ccc agg atg ctc acc ttc aaa ttt tac ttg
       R   M   E   N   Y   R   N   L   K   L   P   R   M   L   T   F   K   F   Y   L   080
241  ccc aag cag gcc aca gaa ttg aaa gat ctt cag tgc cta gaa gat gaa ctt gga cct ctg
       P   K   Q   A   T   E   L   K   D   L   Q   C   L   E   D   E   L   G   P   L   100
301  cgg cat gtt ctg gat ttg act caa agc aaa agc ttt caa ttg gaa gat gct gag aat ttc
       R   H   V   L   D   L   T   Q   S   K   S   F   Q   L   E   D   A   E   N   F   120
361  atc agc aat atc aga gta act gtt gta aaa cta aag ggc tct gac aac aca ttt gag tgc
       I   S   N   I   R   V   T   V   V   K   L   K   G   S   D   N   T   F   E   C   140
421  caa ttc gat gat gag tca gca act gtg gtg gac ttt ctg agg aga tgg ata gcc ttc tgt
       Q   F   D   D   E   S   A   T   V   V   D   F   L   R   R   W   I   A   F   C   160
481  caa agc atc atc tca aca agc cct caa taa
       Q   S   I   I   S   T   S   P   Q   Z
```

Figure 3.3 Nucleotide and derived amino acid sequences of the cDNA encoding the mIL-4 (A) and mIL-2 (B) genes. The sequences are completely identical to the corresponding published sequences.

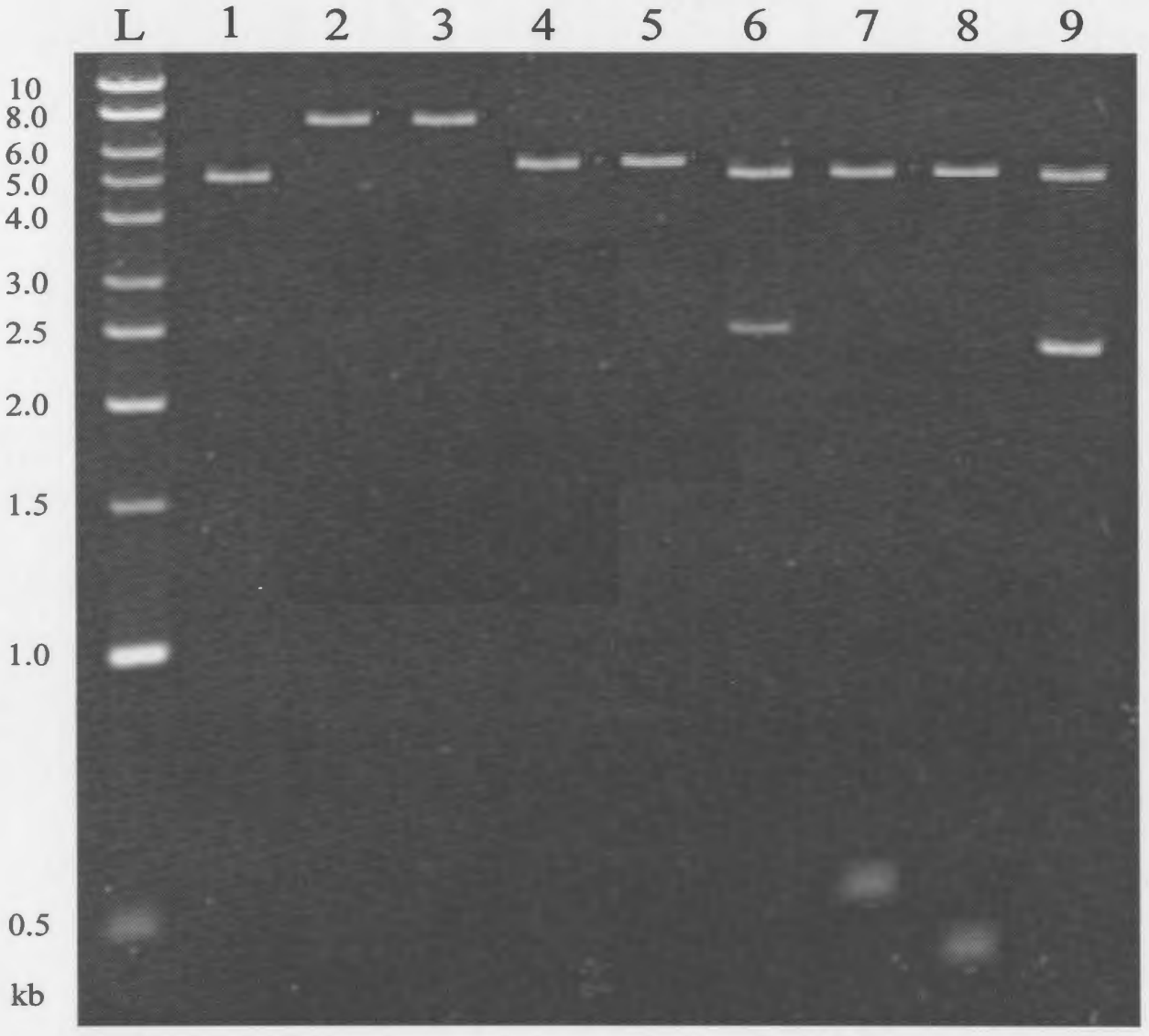


Figure 3.4. Restriction enzyme analysis of pcDNA3.1zeo$^+$ alone or containing hTSHR, mTSHR, mIL-2 or mIL-4 cDNA inserts. *KpnI* digested each of the five plasmids yielding the actual size of each plasmid: pcDNA3.1zeo$^+$ = 5015 bp (lane 1), pcDNA3.1zeo$^+$/hTSHR = 7428 bp (lane 2), pcDNA3.1zeo$^+$/mTSHR = 7249 bp (lane 3), and pcDNA3.1zeo$^+$/IL-2 = 5469 bp (lane 4), and pcDNA3.1zeo$^+$/mIL-4 = 5382 bp (lane 5). The hTSHR insert (~2.5 kb) was released when digested with *XhoI* (lane 6), while mIL-2 (~ .5 kb), mIL-4 (~.4 kb), and mTSHR (~2.3 kb) cDNA inserts were released when digested with both *KpnI* and *XbaI* (lane 7-9 respectively).

3.3.3 Transfection Efficiency of CHO Cells: Luciferase Assay

Transfection efficiency of CHO cells was determined using the luciferase reporter gene system. Cell lysates of CHO cells transiently transfected with varying amounts of either pGL3-Basic or pGL3-Control vectors were mixed with luciferase assay reagent (substrate) and light intensity was measured using a luminometer. As shown in Figure 3.5, cell lysates from CHO cells transiently transfected pGL3-Control, but not pGL3-Basic vectors, contain the luciferase gene product based on the its ability to produce light following the addition of luciferin substrate. The inability of pGL3-Basic transfected CHO cells to produce light following substrate addition was expected due to the lack of eukaryotic promoter and enhancer sequences preventing luciferase gene expression in mammalian cells. No difference in light intensity was observed when cells were transfected with 0.25 or 0.5 μg of DNA. These results confirm CHO cells ability to be transfected with plasmid DNA.

3.3.4 Expression and Functionality of hTSHR and mTSHR

Expression and functionality of hTSHR and mTSHR cDNA inserts were tested by measuring the cAMP response of CHO cells transiently transfected with pcDNA3.1zeo$^+$ containing either the hTSHR or mTSHR insert following stimulation with the TSHR agonist, TSH. CHO cells stably expressing native hTSHR (JP09 cells)[30] were used as a positive control. Following bTSH stimulation, an increase in cAMP concentration was observed in JP09 as well as CHO cells transiently transfected with either pcDNA3.1zeo$^+$/hTSHR or pcDNA3.1zeo$^+$/mTSHR (Figure 3.6). Under similar conditions, no cAMP response was observed from CHO cells transiently transfected with

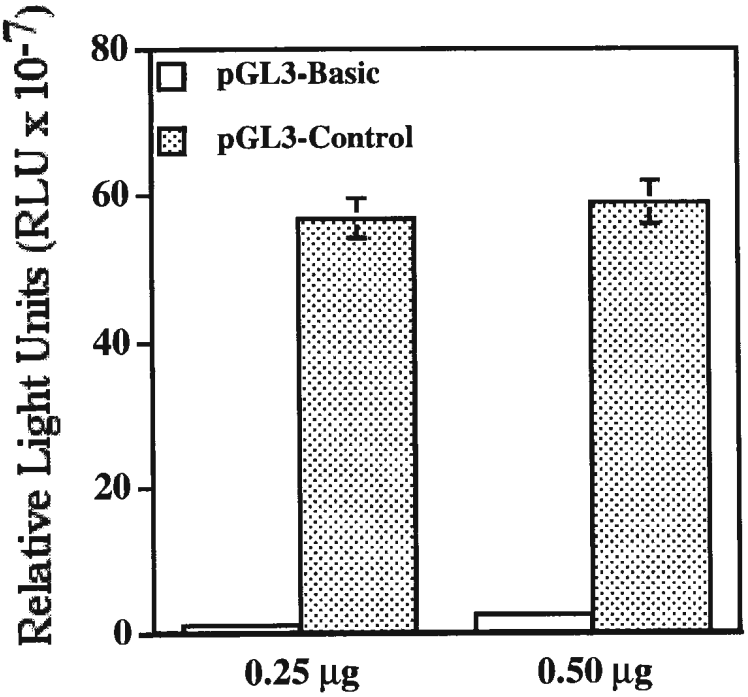


Figure 3.5. Luciferase gene expression in CHO cells. Cell lysates (20 µl) of CHO cells transiently transfected with either pGL3-Basic or pGL3-Control vectors (0.25 µg or 0.5 µg) were mixed with luciferin substrate, and the light produced following the reaction between luciferase and luciferin was measured using a Monolight® 2010 luminometer. Results are expressed as mean of duplicates ± SD.

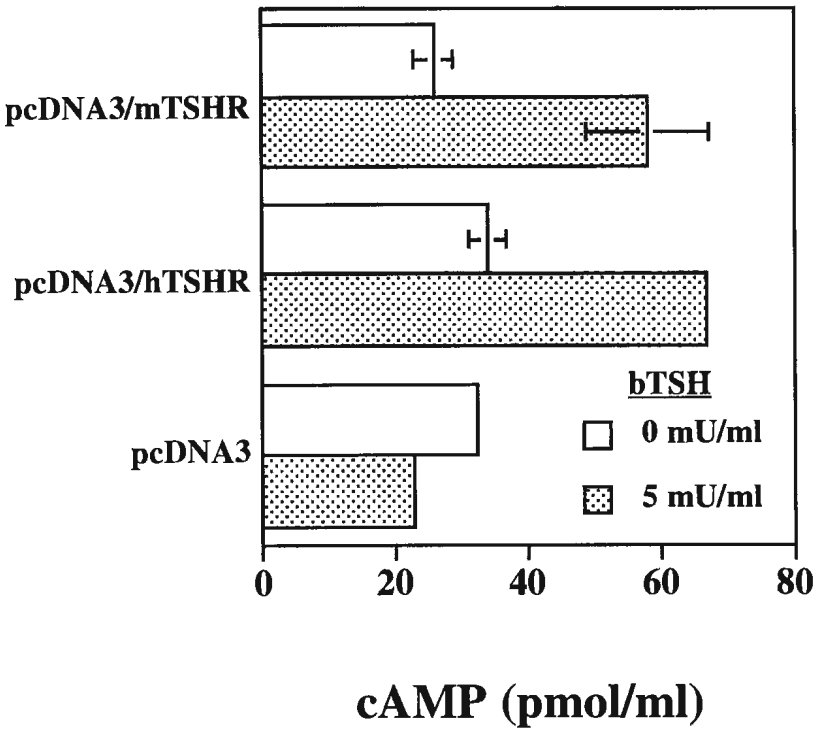


Figure 3.6. Bovine TSH-induced cAMP responses of CHO-K1 cells transiently transfected with pcDNA3.1zeo$^+$ alone or pcDNA3.1zeo$^+$ containing the hTSHR or mTSHR cDNA insert. Cells were stimulated in the presence or absence of 5 mU/ml bTSH for 2 hr, lysed, and cell lysates assayed for total cellular cAMP using a commercial enzymeimmunoassay kit. A 7-fold cAMP increase was observed when CHO stably expressing native hTSHR (JP09 cells) were stimulated with bTSH (data not shown).

the empty vector. Although all cells produced similar baseline levels of cAMP, JP09 cells produced significantly higher amounts of cAMP following bTSH stimulation compared to CHO cells. The reason for this is uncertain, but may be attributed to the high level of hTSHR protein expressed on JP09 cells (90 000 molecules/cell) compared to the unknown level of receptor expression on transiently transfected CHO cells.

3.3.5 Expression and Functionality of mIL-2 and mIL-4

A CT.4S and CTLL-2 proliferation bioassay confirmed the expression and functionality of IL-4 and IL-2 released in tissue culture supernatants from pcDNA3.1zeo$^+$/mIL-4 and pcDNA3.zeo$^+$/mIL-2-transfected CHO cells (Figure 3.7A & B respectively). Supernatants from CHO cells transfected with the empty vector (pcDNA3.1zeo$^+$) were devoid of IL-4 or IL-2 activity.

3.3.6 Specificity of TSHR antibodies for GPI9-5 cells

CHO cells stably expressing GPI-anchored hTSHR-ECD (GPI9-5) or vector-transfected CHO cells (CHO1) were used to detect TSHR-specific autoantibodies in immune mouse sera by flow cytometry. Prior to analysis of sera, the specificity of TSHR antibodies for GPI9-5 cells was determined using the A10 mAb known to recognize a TSHR-ECD peptide (a.a. 22-35). As shown in Figure 3.8, no shift in the mean channel fluorescence (MCF) was observed when GPI9-5 or CHO1 cells were incubated in the presence of the HB65 mAb specific for the nucleoprotein of the influenza type A virus or when CHO1 cells were incubated in the presence of A10 mAb. However, a large shift was observed when GPI9-5 cells were incubated with A10 mAb. These results confirmed TSHR-ECD expression on GPI9-5 cells. For normalization purposes, in subsequent

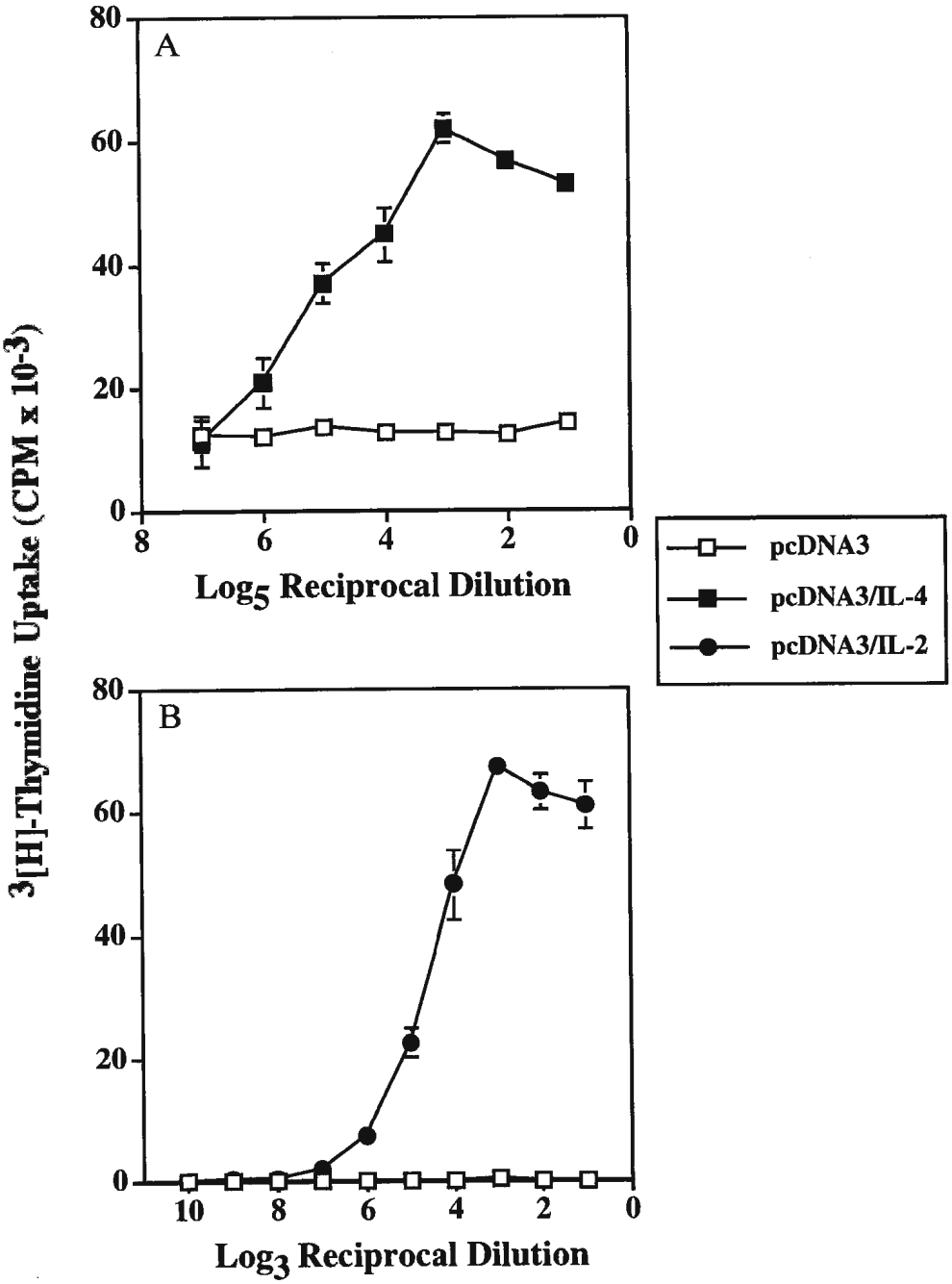


Figure 3.7 Expression and functionality of mIL-4 (A) and mIL-2 (B). Supernatants collected from CHO cells transiently transfected with pcDNA3.1zeo$^+$ alone or pcDNA3.1zeo$^+$ containing the IL-4 (A) or IL-2 (B) cDNA insert were serially diluted and incubated with 10^4 CTLL-2 or CT.4S cells for 24 or 48 hours respectively. Twenty four (CT.4S) or eight hours (CTLL-2) prior to harvesting, ^{3}[H] thymidine was added to each well (1 μCi/well). Cells were harvested using the Harvester 96® Mach III M and incorporated radioactivity was measured using the TopCount NXT™ Microplate Scintillation & Luminescence Counter. Results are expressed as mean of triplicates ± SD.

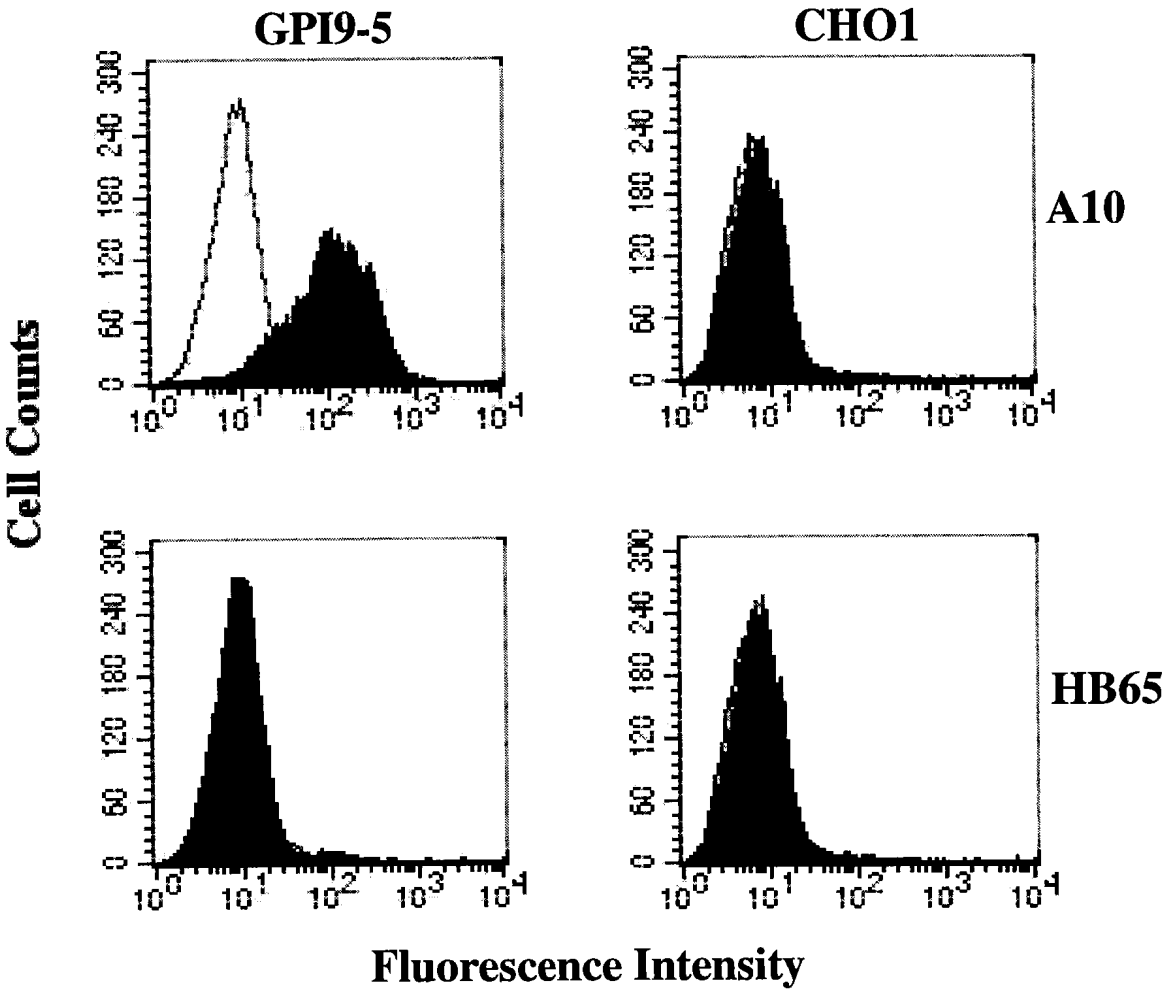


Figure 3.8 Detection of GPI-anchored hTSHR-ECD expressed on CHO cells (GPI9-5). GPI9-5 and empty vector transfected CHO cells (CHO1) (2×10^5 cells/tube) were incubated in the presence (black histogram) or absence (white histogram) of TSHR-specific (A10) or non-specific (HB65) mAb (10 μg/ml), washed, and subsequently incubated with fluorescein-conjugated γ-chain-F_c-specific goat anti-mouse IgG (1:80). Fluorescence of 10 000 cells were analyzed using a FACStar Plus analyzer and CellQuest software.

experiments results were expressed as the MCF ratio between GPI9-5 and CHO1 cells and values greater than 3SD above the control mean were considered positive.

3.3.7 Intradermal DNA immunization with heterologous TSHR induces TSHR-specific autoantibody production in BALB/cJ mice

To determine whether i.d. DNA immunization of BALB/cJ with pcDNA3.1zeo$^+$ encoding the heterologous or homologous TSHR could induce an immune response against the TSHR molecule, individual serum samples were tested for: a) TSHR-specific antibodies, b) TSAb activity, c) elevated TT4, and d) lymphocytic infiltration of the thyroid gland. As shown in Table 3.1, sera from 4 out of the 8 BALB/cJ mice (50 %) immunized 3x with 10 μg of pcDNA3.1zeo$^+$/hTSHR contained TSHR-specific autoantibodies in week 8 and 10. One of these 4 mice (mouse no. 7), however, exhibited only marginal antibody production with GPI9-5/CHO1 MCF ratios (2.08 and 1.92) only slightly greater than control values (mean + 3SD = 1.83 and 1.54). The other three mice had GPI9-5/CHO1 MCF ratios between 7.56 and 85.72; up to a 45-fold increase compared to control MCF ratios. In contrast, when BALB/cJ mice were immunized with pcDNA3.1zeo$^+$ containing the homologous TSHR insert (mTSHR), only 1 mouse was positive for TSHR antibodies in week 8 (mouse no. 7), and 3 were positive in week 10 (mouse no. 4, 7, and 8). Once again, however, the MCF ratios of these mice were only marginally greater than control values. Of the mice that were positive for TSHR antibodies, none had detectable TSAb activity or elevated TT4 levels. Furthermore, thyroid size and morphology were comparable with thyroids of control mice, and no lymphocytic infiltration was observed (data not shown). These results confirm the efficacy of intradermal DNA immunization to induce a humoral immune response in

Table 3.1 Comparison of the humoral immune response (TSHR-specific Ab production) and thyroid function of BALB/cJ mice following i.d. DNA immunization with varying doses of homologous (murine) and heterologous (human) TSHR antigen

Antigen (Dose)[a]	Mouse No.	GPI9-5/CHO1 MCF Ratio[b]		cAMP (pmol/ml)[c]		TT4 (μg/dl)[d]	
		Week 8	*Week 10*	*Week 8*	*Week 10*	*Week 8*	*Week 10*
E (10 μg)	1	1.66	1.44	18.5	27.4	4.80	5.69
	2	1.58	1.39	17.5	21.8	5.44	5.26
	3	1.55	1.45	21.5	20.1	6.12	5.83
	4	1.50	1.34	19.0	22.5	5.20	6.13
	5	1.36	1.43	17.9	21.7	4.98	5.29
	6	1.46	1.36	21.4	25.4	6.27	6.56
	[redacted]						**7.29**
M (10 μg)	1	1.79	1.41	19.5	20.7	5.57	5.97
	2	1.57	1.40	22.5	20.7	4.87	6.05
	3	1.54	1.29	19.8	21.3	6.58	4.95
	4	1.61	**1.88**	20.0	19.7	4.86	5.93
	5	1.64	1.11	19.2	18.2	5.74	5.51
	6	1.78		21.9		6.83	
	7	**2.04**	**1.57**	18.2	20.7	4.66	5.84
	8	1.72	**1.59**	19.4	18.6	5.37	6.16
H (10 μg)	1	1.52	1.38	21.2	18.9	6.83	6.58
	2	1.68	1.40	22.6	27.2	5.23	6.49
	3	1.53	1.37	24.2	26.7	6.29	6.23
	4	1.67	1.47	20.0	21.8	6.44	6.36
	5	**30.83**	**18.49**	18.2	21.1	5.00	5.87
	6	**7.66**	**7.56**	16.9	24.3	5.16	6.18
	7	**2.08**	**1.92**	17.3	23.6	4.48	6.06
	8	**85.72**	**69.51**	19.5	29.0	5.83	6.05
E (50 μg)	1	1.63	1.59	17.7	18.9	4.93	5.24
	2	1.46	1.43	19.2	23.7	5.97	5.86
	3	1.48	1.40	21.6	21.7	5.21	6.46
	4	1.54	1.34	22.2	21.9	6.14	6.50
	5	2.84	1.95	22.5	23.8	4.96	6.47
	6	1.46	1.38	31.0	28.6	4.65	5.39
	[redacted]						**7.71**
M (50 μg)	1	1.77	1.63	18.0	22.2	4.95	5.48
	2	1.74	1.50	24.5	27.3	6.73	4.70
	3	1.97	1.40	25.4	26.8	5.71	5.65
	4	1.55	1.42	18.7	21.7	5.70	6.57
	5	2.10	1.49	20.4	23.9	5.61	6.47
	6	2.08	1.58	22.0	23.5	5.72	6.12
	7	1.43	1.44	22.4	26.3	5.76	5.80
	8	3.09	2.13	24.4	25.0	5.66	5.24

[a]BALB/cJ mice were immunized i.d. with the indicated plasmid and dose as described in Materials and Methods. E = pcDNA3.1zeo$^+$ alone, H and M = pcDNA3.1zeo$^+$ containing the hTSHR and mTSHR cDNA insert respectively. Mice were boosted with the same amount of DNA 3 and 6 weeks postpriming.

[b]TSHR-specific autoantibodies were detected in immunized mouse sera by flow cytometry as described in Materials and Methods. Results are expressed as the MCF ratio between GPI9-5 and CHO1 cells.

[c]JP09 cells were stimulated with immune mouse serum (1:10) for 2 hours, and total cAMP was measured as described in Materials and Methods.

[d]An RIA kit was used for TT4 determination as described in Materials and Methods.

[e]Values greater than control mean + 3SD were considered positive and are represented in **bold** typeface.

BALB/cJ mice against the antigen (TSHR) encoded in the plasmid vector. Nonetheless, the antibodies produced against the TSHR lacked biological activity since the TT4 levels in these mice remained normal. Moreover, a stronger immune response was observed following immunization with the heterologous rather than the homologous TSHR, based on the larger proportion of mice producing TSHR antibodies (50%), as well as the higher GPI9-5/CHO1 MCF ratios.

To test whether an increase of the immunizing DNA dose would enhance the immune response (i.e. induce TSHR-Ab in a larger proportion of mice) against the homologous TSHR, BALB/cJ mice were challenged with 5x more pcDNA3.1zeo^{+}/mTSHR (50 μg) per immunization. As shown in Table 3.1, none of the mice produced antibodies that could bind to the hTSHR-ECD expressed on CHO cells (GPI9-5 cells), correlating with the lack of TSAb activity and the presence of normal TT4 serum levels and normal thyroid size and histology.

3.3.8 Attempts to modulate the immune response through co-delivery of cytokine plasmid vectors

Several research groups have used plasmid DNA encoding various cytokines to enhance or bias the immune response generated by DNA vaccination (reviewed in Gurunathan et al.[103]). In an effort to bias the immune response toward a Th2 response and promote Ab production, BALB/cJ mice were co-injected with plasmid DNA containing the mTSHR cDNA insert together with the plasmid encoding the mIL-4 cytokine. Surprisingly, none of the mouse sera contained TSHR-specific antibodies, detectable TSAb activity, or elevated TT4 levels (Table 3.2). Histologic examination of the thyroids also appeared normal (data not shown). Similar results were obtained while attempting to

Table 3.2 Effect of mIL-4 cytokine co-delivery on TSHR-specific Ab production and thyroid function in BALB/cJ mice

Antigen (Dose)[a]	Mouse No.	GPI9-5/CHO1 MCF Ratio[b]			cAMP (pmol/ml)[c]			TT4 (µg/dl)[d]		
		W8	*W10*	*W12*	*W8*	*W10*	*W12*	*W8*	*W10*	*W12*
M (50 µg)	**1**	1.77	1.63		18.0	22.2		4.95	5.48	
	2	1.74	1.50		24.5	27.3		6.73	4.70	
	3	1.97	1.40		25.4	26.8		5.71	5.65	
	4	1.55	1.42	ND	18.7	21.7	ND	5.70	6.57	ND
	5	2.10	1.49		20.4	23.9		5.61	6.47	
	6	2.08	1.58		22.0	23.5		5.72	6.12	
	7	1.43	1.44		22.4	26.3		5.76	5.80	
	8	3.09	2.13		24.4	25.0		5.66	5.24	
M (50 µg) + IL-4 (50 µg)	**1**	2.09	1.45		27.3	22.5		7.01	6.05	
	2	2.03	1.58		33.4	23.3		6.14	6.39	
	3	2.02	1.42		17.1	24.3		5.74	6.50	
	4	1.67	1.41	ND	20.6	20.0	ND	6.23	6.48	ND
	5	1.50	1.55		17.7	24.9		4.67	5.86	
	6	1.56	1.65		19.2	22.7		4.56	6.18	
	7	1.69	1.48		17.4	23.0		4.48	5.86	
	8	1.67	1.60		21.4	23.9		5.96	6.54	
E (50 µg) + IL-4 (50 µg)	**1**	1.40	1.82	2.08	20.7	36.5	36.2	5.90	5.09	5.11
	2	1.51	1.70	2.07	19.1	35.3	34.7	6.25	5.50	5.86
	3	1.42	1.76	1.64	21.4	31.0	29.3	6.17	5.93	6.31
	4	1.40	1.69	1.79	21.3	33.9	34.0	5.43	6.51	5.21
	5	1.45	1.55	2.37	27.8	31.2	31.9	6.14	5.85	5.37
	6	1.37	1.58	1.86	29.5	36.1	37.2	6.15	5.19	5.94
	7	1.18	1.48	1.64	24.5	37.8	36.2	6.10	5.15	6.17
[illegible]									[illegible]	
H (50 µg)	**1**	**6.46**	**21.02**	**10.05**	20.6	**49.5**	38.6	5.36	5.48	5.33
	2	**6.40**	**26.33**	**11.98**	24.7	34.5	31.0	**13.49**	**13.40**	**11.12**
	3	**10.96**	**56.64**	**22.69**	17.5	30.8	28.0	6.36	6.11	6.19
	4	1.52	1.67	2.26	18.3	25.7	20.3	6.13	5.31	4.74
	5	1.07	1.28	1.69	20.3	24.2	24.6	6.01	5.58	4.93
	6	**38.62**			27.5			**9.80**		
	7	**10.24**	**30.69**		34.8	22.1		**7.73**	**16.21**	
	8	1.56	1.95	2.40	30.5	30.5	22.7	5.06	5.06	4.38
H (50 µg) + IL-4 (50 µg)	**1**	**1.78**	1.34	2.55	30.7	32.7	21.9	5.51	5.38	4.72
	2	1.32	1.78	2.34	28.1	36.1	26.0	4.41	4.50	5.23
	3	**1.82**	**2.23**	2.42	23.5	27.7	31.4	5.60	4.72	4.27
	4	1.36	**2.16**	2.17	24.5	23.8	26.2	6.15	5.74	5.56
	5	1.51	1.91	2.09	25.3	22.6	26.4	5.53	5.76	5.90
	6	**1.81**	1.62	1.94	26.4	22.1	18.0	5.54	5.26	5.60
	7	**1.73**	**3.16**	**2.85**	31.3	20.3	18.0	6.28	6.52	6.47

[a]BALB/cJ mice were immunized i.d. with the indicated plasmid and dose as described in Materials and Methods. E = pcDNA3.1zeo$^+$ alone, H, M, and IL-4 = pcDNA3.1zeo$^+$ containing the hTSHR, mTSHR, and mIL-4 cDNA insert respectively. Mice were boosted with the same amount of DNA 3 and 6 weeks postpriming.

[b]TSHR-specific autoantibodies were detected in immunized mouse sera by flow cytometry as described in Materials and Methods. Results are expressed as the MCF ratio between GPI9-5 and CHO1 cells.

[c]JP09 cells were stimulated with immune mouse serum (1:10) for 2 hours, and total cAMP was measured as described in Materials and Methods.

[d]An RIA kit was used for TT4 determination as described in Materials and Methods.

[e]Values greater than control mean + 3SD were considered positive and are represented in **bold** typeface.

enhance the immune response through co-injection of pcDNA3.1zeo$^+$/mTSHR together with pcDNA3.1zeo$^+$ containing the mIL-2 gene (Table 3.3). Based on these results, as well as those obtained in the previous experiment, it appears that mice immunized with a high dose of pcDNA3.1zeo$^+$/mTSHR are not induced to respond against the self-antigen regardless of co-delivery of cytokine vectors.

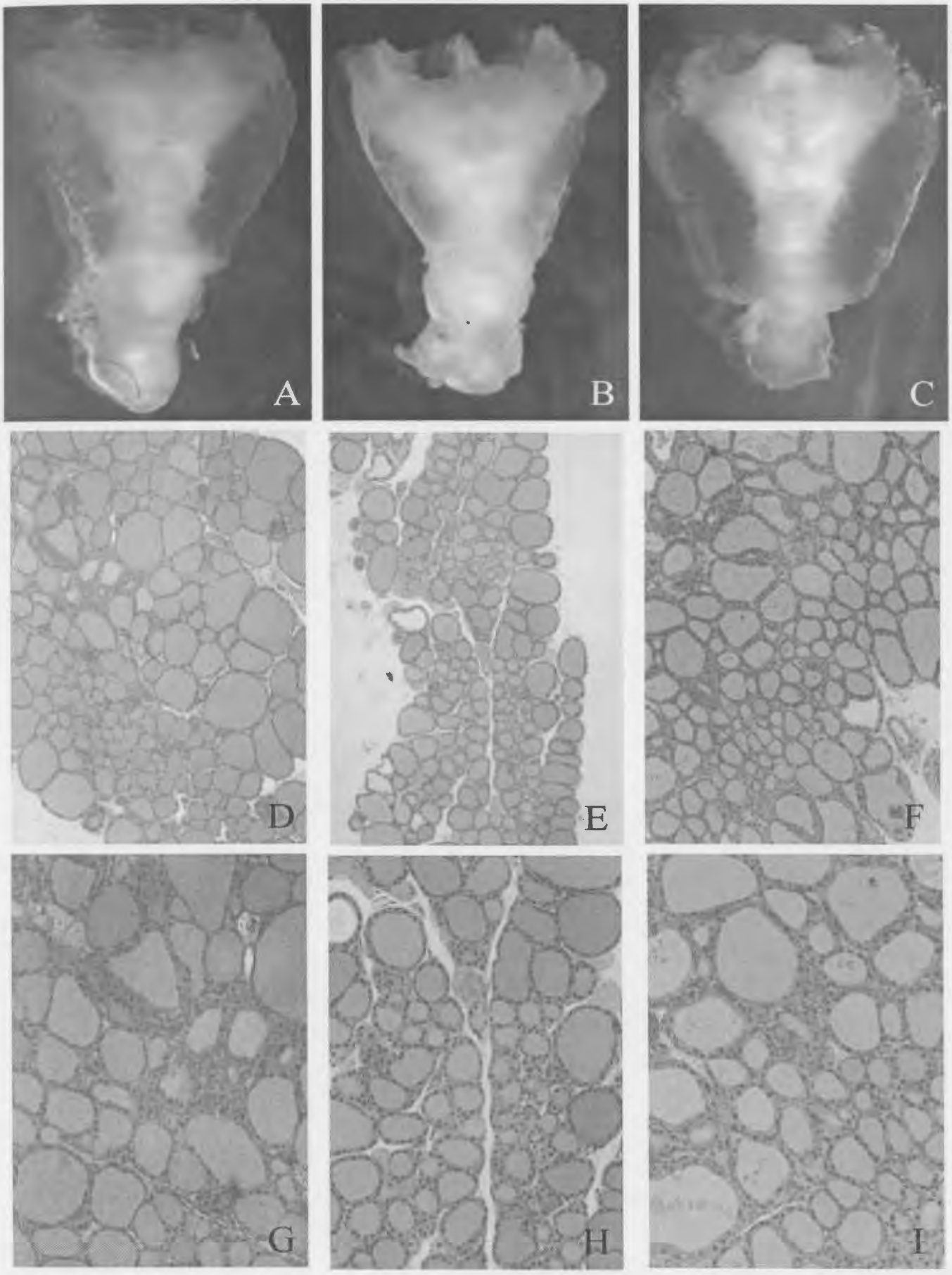
Experiments based on co-delivery of cytokine vectors were also performed using the heterologous hTSHR, and because the larger dose (50 μg of DNA/immunization) of pcDNA3.1zeo$^+$/hTSHR had not been tested in the previous experiment, BALB/cJ mice were also immunized with 50 μg of pcDNA3.1zeo$^+$/hTSHR alone. Interestingly, immunization with the larger dose of pcDNA3.1zeo$^+$/hTSHR, not only induced TSHR-antibody production in a larger proportion of mice (62.5 %, week 8), but 3 of these mice (mouse no. 2, 6, and 7) exhibited elevated TT4 levels (Table 3.2). The TT4 levels for mouse no. 7 in week 10, for example, almost exceeded 3x the normal range (16.21 μg/dl compared to 5.09-6.51 μg/dl). Unfortunately, 2 of the 3 thyrotoxic mice died following blood collection in week 8 (mouse no. 6) and week 10 (mouse no. 7) and therefore no data was obtained for these mice in subsequent weeks. However, antibody detection and elevated TT4 levels in the remaining hyperthyroid mouse (no. 2) persisted for the duration of the experiment (12 weeks). In addition, thyroid glands from 2 of the 3 thyrotoxic mice (mouse no. 2 and 7) showed marked hypertrophy (Figure 3.9A and C respectively) and exhibited follicular hyperplasia and thyrocyte hypercellularity (Figure 3.9D, F, G, and I) compared to control thyroids (Figure 3.9B, E, and H). Yet the marked increase in serum TT4 in 3 of the 8 BALB/cJ mice immunized with pcDNA3.1zeo$^+$/

Table 3.3 Effect of mIL-2 cytokine co-delivery on TSHR-specific Ab production and thyroid function in BALB/cJ mice

Antigen (Dose)[a]	Mouse No.	GPI9-5/CHO1 MCF Ratio[b]		cAMP (pmol/ml)[c]		TT4 (μg/dl)[d]	
		Week 8	*Week 10*	*Week 8*	*Week 10*	*Week 8*	*Week 10*
E (50 μg)	**1**	1.13	1.24	22.7	20.1	6.30	6.03
+	**2**	1.04	1.28	21.0	26.3	6.29	6.05
IL-2 (50 μg)	**3**	1.10	1.13	17.0	25.1	6.17	6.17
	4	1.10	1.22	15.4	26.5	5.73	6.43
	5	1.16	1.26	17.3	19.4	5.88	5.89
	6	1.05	1.10	16.8	18.7	4.96	6.44
	7	1.17	1.24	19.2	24.5	5.88	5.79
	8	1.05	1.37	21.6	21.1	5.59	5.60
Mean + 3SD		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	**6.93**
H (50 μg)	**1**	1.13	1.12	24.3	32.3	5.70	6.54
+	**2**	**1.27**	1.36	22.4	22.7	6.70	6.58
IL-2 (50 μg)	**3**	**1.28**	1.31	17.3	24.0	6.98	6.11
	4	1.16	1.17	18.1	20.4	5.59	5.95
	5	**1.88**	1.28	20.3	16.1	6.81	5.89
	6	1.18	1.04	18.2	16.8	6.35	5.57
	7	1.15	1.07	16.7	18.5	6.22	6.07
	8	1.09	1.10	22.9	22.9	6.03	5.99
M (50 μg)	**1**	**1.33**	1.18	28.3	28.5	6.50	5.99
+	**2**	1.12	1.12	22.2	27.1	6.52	5.74
IL-2 (50 μg)	**3**	1.11	1.08	22.1	29.2	5.82	6.10
	4	1.14		19.0		5.68	
	5	**1.54**	1.14	23.1	29.9	6.47	6.03
	6	1.15	1.09	22.0	29.9	6.03	5.88
	7	**1.43**	1.03	18.1	26.7	5.13	6.03
	8	0.75	0.85	22.5	28.4	5.98	5.91

[a]BALB/cJ mice were immunized i.d. with the indicated plasmid and dose as described in Materials and Methods. E = pcDNA3.1zeo$^+$ alone, H, M, and IL-2 = pcDNA3.1zeo$^+$ containing the hTSHR, mTSHR, and mIL-2 cDNA insert respectively. Mice were boosted with the same amount of DNA 3 and 6 weeks postpriming.
[b]TSHR-specific autoantibodies were detected in immunized mouse sera by flow cytometry as described in Materials and Methods. Results are expressed as the MCF ratio between GPI9-5 and CHO1 cells.
[c]JP09 cells were stimulated with immune mouse serum (1:10) for 2 hours, and total cAMP was measured as described in Materials and Methods.
[d]An RIA kit was used for TT4 determination as described in Materials and Methods.
[e]Values greater than control mean + 3SD were considered positive and are represented in **bold** typeface.

Figure 3.9 Gross and histological examination of BALB/cJ mouse thyroids following genetic immunization. Thyroid glands of control (B) and hyperthyroid mice (A & C) immunized with pcDNA3.1zeo^{+} alone (mouse no. 1, Table 3.1) or pcDNA3.1zeo^{+} containing the hTSHR cDNA insert (mouse no. 2 and 7, Table 3.2) respectively. Hematoxylin and eosin-stained methacrylate sections of thyroids from normal (E & H) or hyperthyroid (D, F, G, and I) mice (D-F, 1000x; G-I, 2000x). Note the enlarged follicles (follicular hyperplasia) and the columnar shape of follicular cells with enlarged nuclei from hyperthyroid mice.


A
B
C
D
E
F
G
H
I

hTSHR and the gross and histological changes in 2 of these 3 thyrotoxic thyroids, did not correlate with TSAb activity (Table 3.2). The serum obtained from mouse no. 1 within the same group, however, stimulated JP09 cells to produce significantly higher cAMP concentrations (49.5 pmol/ml) than sera from control mice previously immunized with the empty vector and pcDNA3.1zeo$^+$/mIL-4 (control mean + 3SD = 42.4 pmol/ml). Unfortunately, this TSAb activity was only detected in week 10 and also did not correlate with elevated T4 levels.

Encouraged by these results, we proceeded to co-inject BALB/cJ mice with the same dose of pcDNA3.1zeo$^+$/hTSHR together with pcDNA3.1zeo$^+$ encoding mIL-4 cytokine. Co-delivery of pcDNA3.1zeo$^+$/hTSHR together with pcDNA3.1zeo$^+$/mIL-4, however, did not induce hyperthyroidism or goiter in any mice. Only a few mice produced antibodies specific for the TSHR, and those that did had GPI9-5/CHO1 MCF ratios only slightly above MCF ratios obtained from mice immunized with the empty vector and pcDNA3.1zeo$^+$/mIL-4. Moreover, mice that were positive for TSHR antibodies in week 8 were not necessarily positive in week 10 and 12 and vice versa (Table 3.2). Comparable results were obtained when BALB/cJ mice were co-injected with pcDNA3.1zeo$^+$/hTSHR and plasmid encoding mIL-2 (Table 3.3). These results do not support previous findings that co-delivery of cytokine-encoding DNA can enhance or bias the immune response generated by DNA vaccination[127-132].

3.3.9 TSHR-specific antibody production in AKR/J mice following i.d. immunization with pcDNA3.1zeo$^+$/hTSHR

To test whether intradermal DNA immunization with pcDNA3.1zeo$^+$/hTSHR could also induce hyperthyroidism in a different mouse strain, AKR/J (H-2^k) mice were immunized

3x with 50 μg of pcDNA3.1zeo^{+}/hTSHR. As shown in Table 3.4, four out of the seven (57 %) immunized mice (mouse nos. 3, 4, 5, and 7) were consistently positive for TSHR-specific antibodies in week 8, 10 and 12. Two additional mice (nos. 2 and 6) were positive in week 10 only, but their GPI9-5/CHO1 ratios were only marginally higher (1.87 and 1.99) when compared to control mice (control mean + 3SD = 1.80). None of the mice contained TSAb activity in their sera, although the TT4 level of one mouse (no. 6) was significantly higher (3.79 μg/dl) in week 10 than the normal range (1.81-2.97 μg/dl). However, unlike BALB/cJ mice where elevated TT4 levels corresponded with thyroid hypertrophy, follicular hyperplasia, and thyrocyte hypercellularity, gross and histological analysis of the thyroid obtained from this thyrotoxic mouse (no. 6), was comparable with control mouse thyroids (data not shown). This was not surprising since at the time of thyroid removal (week 12); mouse no. 6 was no longer thyrotoxic. These results confirm that a humoral immune response against the TSHR molecule can also be induced in a different MHC haplotype (H-2^{k}) following intradermal DNA immunization with pcDNA3.1zeo^{+}/hTSHR. However, AKR/J mice appear less susceptible to hyperthyroidism than BALB/cJ based on the lower proportion of mice (1/7 versus 3/8) with elevated TT4 levels, as well as the lack of gross and histological features of thyroid hyperactivity.

Efforts to modulate the immune response through co-delivery of cytokine-plasmid vectors were also examined in AKR/J mice. Sera from 3 out of the 7 AKR/J mice (mouse nos. 1, 2 and 4) co-injected with pcDNA3.1zeo^{+}/hTSHR and pcDNA3.1zeo^{+}/IL-4 contained antibodies that recognized the TSHR-ECD expressed on CHO cells (GPI9-5)

Table 3.4 TSHR-specific Ab production and thyroid function of AKR/J mice following i.d. DNA immunization with pcDNAzeo$^+$/hTSHR alone or together with pcDNA3.1zeo$^+$/mIL-4

Antigen (Dose)[a]	Mouse No.	GPI9-5/CHO1 MCF Ratio[b]			cAMP (pmol/ml)[c]			TT4 (μg/dl)[d]		
		W8	*W10*	*W12*	*W8*	*W10*	*W12*	*W8*	*W10*	*W12*
E (50 μg)	**1**	1.55	1.44	3.48	35.3	27.1	32.5	2.64	2.64	2.76
+	**2**	1.69	1.41	2.13	31.4	27.2	35.2	2.37	1.81	2.19
IL-4 (50 μg)	**3**	1.13	1.48	2.23	30.7	24.6	27.6	2.74	2.97	2.25
	4	1.50	1.68	2.42	26.5	36.8	29.6	2.49	2.36	3.64
	5	1.60	1.52	2.51	26.9	27.9	34.7	2.22	2.19	1.86
	6	1.87	1.61	2.34	31.6	29.7	34.1	2.72	2.54	2.27
	7	1.69	1.47	1.48	30.1	25.3	30.4	2.30	2.36	2.71
	8	1.72	1.38	1.82	38.6	27.7	36.1	1.75	2.14	2.48
[redacted]										4.14
H (50 μg)	**1**	1.68	1.40	1.76	32.9	31.3	40.7	2.30	2.74	3.45
	2	1.57	**1.87**	2.32	33.5	32.7	39.3	2.29	2.92	2.57
	3	**4.84**	**28.25**	**10.26**	32.5	36.1	35.8	2.35	2.41	2.63
	4	**9.78**	**37.01**	**16.52**	38.0	38.7	31.5	2.46	2.80	2.95
	5	**2.30**	**3.41**	**4.53**	39.8	35.5	37.8	2.69	2.98	3.34
	6	1.62	**1.99**	3.16	40.9	27.5	39.3	2.55	**3.79**	3.35
	7	**3.46**	**27.74**	**7.26**	39.2	30.4	37.4	2.41	2.29	2.63
H (50 μg)	**1**	**2.64**	1.64	1.95	36.3	27.6	36.8	**3.42**	2.59	2.80
+	**2**	**5.14**	**34.51**		43.1	32.0		3.20	1.84	
IL-4 (50 μg)	**3**	1.81	1.24	2.03	36.4	27.9	24.0	2.24	1.99	3.53
	4	**3.91**	**8.17**	**5.37**	39.3	35.5	26.4	3.11	3.09	2.89
	5									
	6	1.82	**1.92**	2.01	41.4	33.5	38.4	3.35	2.71	2.72
	7	2.21	**1.92**	2.28	36.9	37.9	27.2	3.32	2.89	2.99
	8	1.54	1.45	2.04	37.1	33.3	31.6	3.18	2.91	2.71

[a]AKR/J mice were immunized i.d. with the indicated plasmid and dose as described in Materials and Methods. E = pcDNA3.1zeo$^+$ alone, H and IL-4 = pcDNA3.1zeo$^+$ containing the hTSHR and mIL-4 cDNA insert respectively. Mice were boosted with the same amount of DNA 3 and 6 weeks postpriming.

[b]TSHR-specific autoantibodies were detected in immunized mouse sera by flow cytometry as described in Materials and Methods. Results are expressed as the MCF ratio between GPI9-5 and CHO1 cells.

[c]JP09 cells were stimulated with immune mouse serum (1:10) for 2 hours, and total cAMP was measured as described in Materials and Methods.

[d]An RIA kit was used for TT4 determination as described in Materials and Methods.

[e]Values greater than control mean + 3SD were considered positive and are represented in **bold** typeface.

in week 8 (Table 3.4). One of these 3 mice (no. 1) also had elevated serum TT4 (3.42 μg/dl) the same week, but this elevation did not persist through week 10 and 12, nor did the antibody response. The other two mice, however, maintained antibody production against the TSHR in week 10, but because mouse no. 2 died following blood collection in week 10, only serum from mouse no. 4 was confirmed to contain TSHR antibodies in week 12. Two additional mice (nos. 6 and 7) were positive for TSHR-specific antibodies in week 10 only, but only marginal difference between GPI9-5/CHO1 ratios of these mice (1.92 and 1.92) and control mice (control mean + 3SD = 1.80) was observed. None of the mice, regardless of antibody production or elevated TT4 exhibited gross or histological thyroid features indicative of hyperactivity. These results do not support the concept that co-delivery of mIL-4 DNA with TSHR-encoding plasmid alters the immune response to TSHR in AKR/J mice.

3.4 DISCUSSION

Genetic immunization with naked plasmid DNA was developed primarily to induce protective immunity, especially against infectious pathogens (i.e. malaria and HIV) and cancer for which, to date, there are no available safe and effective vaccines[103]. Vaccination with naked DNA has also been used to protect animals from developing autoimmune disease[97-101;170]. Here we have used DNA immunization in an attempt to abrogate self-tolerance and induce an autoimmune response against TSHR, the known autoantigen in Graves' disease, in an effort to develop an animal model for this disease.

Very few BALB/cJ mice (< 20%) produced autoantibodies specific for the TSHR molecule following i.d. DNA immunization with pcDNA3.1zeo^{+} encoding the

homologous (murine) TSHR, regardless of antigen dose (Table 3.1) or co-delivery of cytokine-vectors (Table 3.2 & 3.3). Moreover, mice that were positive for TSHR-specific antibodies, exhibited only slightly higher GPI9-5/CHO1 MCF ratios than the control MCF ratio + 3SD. Whether antibodies were produced in a larger proportion of these mice and just not detected because the cells used to detect TSHR antibodies expressed heterologous (human) TSHR-ECD remains unclear. Studies using recombinant mTSHR as the immunogen, however, have shown that murine TSHR-specific autoantibodies inhibited up to 78% of the binding of labeled TSH to native hTSHR, indicating mTSHR-specific autoantibodies are capable of binding to heterologous TSHR molecules[67]. The lack of TSAb or elevated T4 levels, together with normal thyroid size and histology in these mice suggests that DNA immunization with murine TSHR was unable to abrogate self-tolerance. Interestingly, tolerance to the mTSHR was broken following i.p. injection of mitomycin-C-treated M12 cells expressing native mTSHR[80]. Mice developed most of the symptoms of Graves' disease including TBII, TSAb activity, elevated T4 levels, and mild lymphocytic infiltration. Differences in the immunization route and antigen form used most likely contribute to these conflicting findings.

Several studies have used DNA immunization to protect or ameliorate autoimmune disease induction in animals. Ruiz, et al.[99], for example showed that immunization of SJL/J mice with DNA encoding the encephalitogenic residues of myelin proteolipid protein, $PLP_{139-151}$, delayed onset of disease, and reduced mean peak disease severity and mean disease score following subsequent induction of EAE with $PLP_{139-151}$ and CFA. The exact mechanisms behind the protection afforded by DNA vaccination are

unclear, but may involve generation of antigen-specific regulatory cells or induction of anergy in autoreactive T cells. Perhaps, the immune response to a DNA vaccine encoding self Ag is different from what is observed with DNA vaccination to foreign Ags.

On the other hand, when BALB/cJ and AKR/J mice were immunized 3x with varying doses of pcDNA3.1zeo^{+} encoding a heterologous TSHR (hTSHR), between 50 and 60% of the mice developed antibodies that recognized GPI-anchored hTSHR-ECD expressed on CHO cells (Table 3.1, 3.2, and 3.4). These results are comparable to those obtained in a previous study where sera from 3/5 (60%) BALB/cJ mice immunized intramuscularly with pcDNAIII containing the hTSHR gene insert, recognized native TSHR by FACS analysis[83]. Pre-treatment of the muscle with cardiotoxin or immunizing mice with the vector prepared in a 25% sucrose solution induced antibodies in 4/4 and 4/5 mice respectively. Notably, twice as much DNA (100 μg) was injected intramuscularly; and JP09 cells, known to express 90 000 hTSHR/cell[145] were used to detect antibodies. In another study, the same intramuscular immunization regime did not generate TSHR-specific antibodies in BALB/c or AKR/J mice as assessed by flow cytometry, even when using CHO cells which express ~20 x greater number hTSHR molecules (~ 2 x 10^6 TSHR/cell) than JP09 cells[85]. The authors speculated that the conflicting data were a result of the different breeding colonies or the pathogen-free animal facility used to house the mice[85]. In both studies, no changes to thyroid hormone levels were observed following intramuscular immunization. In contrast, we were able to induce consistent hyperthyroidism, with TT4 values as high as 16.21 μg/dl (normal range: 5.09-6.51 μg/dl), in 3 of the 8 BALB/cJ mice immunized with the higher dose (50

μg/immunization) of hTSHR cDNA (Table 3.2). In addition, thyroid glands from two of these mice showed marked hypertrophy (Figure 3.9A & C), and histological examination revealed follicular hyperplasia and thyrocyte hypercellularity indicative of thyroid hyperactivity (Figure 3.9D, F, G, I). However, unlike Costagliola who observed signs of thyroiditis in 14 immunized BALB/cJ mice[83], no lymphocytic infiltration was seen in the thyroids of thyrotoxic mice or any other mouse that remained euthyroid.

For many years, it has been clear that two types of functional TSHR autoantibodies exist in Graves' disease patients: TSAb that activate the TSHR and cause hyperthyroidism, and TSBAbs that block TSH binding and action, thereby causing atrophic hypothyroidism[6]. Clearly in GD patients the relative amounts or affinities of these two types of autoantibodies determine the net biological effect on thyroid cell function. In addition, evidence exists for the presence of neutral TSHR autoantibodies in GD patients, which neither activate the TSHR nor block ligand action[4]. Despite the induction of hyperthyroidism in 3 BALB/cJ mice following i.d. immunization with pcDNA3.1zeo^{+}/hTSHR, none of the mice exhibited detectable TSAb activity. Perhaps the inability to detect TSAbs in these hyperthyroid mice has to do with the delicate balance between these different types of TSHR autoantibodies. In other words, TSAb may very well be present in the sera of these thyrotoxic mice, but their actions are masked or prevented by the presence and actions of TSBAb and neutral TSHR autoantibodies. We cannot, however, exclude the possibility that TSAb were not detected in these mice for other, technical reasons such as the cell line used, sera dilution, incubation period, and culture conditions. It was difficult to establish optimal assay conditions based on previous

literature because of the enormous variation of assay conditions among various studies. The most common bioassay for measuring TSAb activity today uses TSHR-expressing CHO cells. Yet, different groups use different cell lines (i.e. 5'3'TR-ECE, JP09, and JP26) all of which express varying numbers of receptors/cell. In 1999, Wallaschofski et al.[171] found that CHO cells expressing higher TSHR numbers (JP09; 90 000 hTSHR molecules/cell) than normal thyroid epithelial cells are less sensitive in detecting TSHR autoantibodies from GD patients than CHO cells that express comparable numbers of TSHR (JP26; 2000 hTSHR molecules/cell). This decreased sensitivity was attributed to the high nonspecific stimulation by normal sera. We used JP09 cells to detect thyroid-stimulating antibodies, seeding 40 000 cells per well of a 96-well plate overnight before the addition of diluted serum (1:10). Other groups, using different cell lines, have detected TSAbs using pooled and protein A-purified IgG[75;172], while others have detected stimulatory antibodies with neat sera diluted as low as 1:70[77]. We chose a serum dilution of 1:10 based on the study by Wallaschofski et al.[171], however it remains to be determined whether TSAb would have been detectable had we purified IgG from the sera or used a higher or lower serum dilution. Nevertheless, our data clearly demonstrate thyroid hyperactivity based on gross and histological examination, as well as elevated TT4. Together, this data argue strongly in favour of TSAb activity, even though we were unable to detect it.

The inherent bias toward a Th1 or Th2 response of a particular immunization method can sometimes be overcome. For instance, Prayaga, et al.[173] was able to reverse the Th2 immune response induced in mice following epidermal gene gun delivery of

plasmid encoding HIV-1 gp120 or influenza nucleoprotein to a Th1-like response through co-administration of plasmids encoding IL-2, IL-7 and IL-12. Cytokines play important roles in the immune and inflammatory responses as the initiators and regulators of the immune network. Interleukin-2, for example, is a Th1 cytokine produced primarily by activated T cells, which plays a critical role in directing cell-mediated immune responses. Interleukin-4, on the other hand, is a Th2 cytokine, and plays an important role in the humoral immune response[174]. Several other groups have used plasmid DNA encoding various cytokines to enhance or bias the immune response generated by DNA vaccination (reviewed by Gurunathan, et al.[103]). Co-administration of DNA immunogens (i.e. HCV core protein, HIV envelope protein, or ovalbumin) with either IL-2 or IL-4 cytokine genes, for example, has been shown to enhance T cell proliferative responses to the respective protein antigen *in vitro*, with cytokine profiles corresponding to Th1 and Th2 phenotypes respectively[127]. Enhancement of overall antibody production in mice was also demonstrated, with a bias toward IgG2a and IgG1 isotypes when the DNA immunogen was co-injected with IL-2 and IL-4 DNA expression constructs, respectively[103;127-132].

The fact that TSAb are of the IgG1 isotype[9] and intrathyroidal lymphocytes of GD patients predominantly produce Th2 cytokines[166], prompted us to examine whether co-delivery of mIL-4-expressing vectors with TSHR-encoding plasmid DNA would bias the response to a Th2-type. Surprisingly, fewer BALB/cJ mice produced TSHR-specific antibodies following co-injection of pcDNA3.1zeo$^+$/hTSHR and pcDNA3.1zeo$^+$/mIL-4, and those that did had GPI9-5/CHO1 MCF ratios only slightly greater than control MCF

ratios (Table 3.2) indicative of a weak immune response. In addition, none of the mice showed signs of hyperthyroidism (i.e. elevated TT4 or changes to thyroid architecture). Comparing the proportion of mice producing TSHR-specific autoantibodies as well as the fold-increase in GPI9-5/CHO1 MCF ratios between experimental and control groups may not be the best approach to measure enhancement of an immune response. A more quantitative approach such as determining specific antibody titers using an ELISA might have been more reliable and would have enabled us to determine whether the immune response was biased toward a Th2-type by measuring the IgG2a:IgG1 ratio frequently used to reflect Th1 and Th2 immune responses. However, due to the inherent difficulties in obtaining appropriately folded TSHR protein, as discussed in Section 1.3.3, we chose to detect TSHR autoantibodies using CHO cells expressing native hTSHR-ECD. Collectively, our data do not support the view that co-injection of hTSHR-encoding and IL-4-encoding plasmids augments the TSHR-specific response. Our attempts to enhance the immune response through co-delivery of mIL-2 cytokine vectors also failed. Once again, fewer BALB/cJ mice produced antibodies specific for the TSHR following co-injection with pcDNA3.1zeo^{+}/hTSHR and pcDNA3.zeo^{+}/mIL-2 (Table 3.3), as compared to pcDNA3.1zeo^{+}/hTSHR alone (Table 3.2), and those that did had low GPI9-5/CHO1 MCF ratios.

It remains unclear why immunization of BALB/cJ mice with hTSHR cDNA alone, but not in conjunction with cytokine-vectors can induce a strong humoral immune response against the TSHR, which sometimes resulted in hyperthyroidism. Perhaps the weaker immune response (as determined by GPI9-5/CHO1 ratios) may be due to the

competition of plasmid uptake after challenge when a mixture of plasmid is used. Co-expressing the TSHR gene together with a cytokine gene using a bicistronic plasmid vector may alleviate such competition if it exists. Further experimentation using a more quantitative approach to measure TSHR-specific autoantibodies may also give us a better understanding the effect cytokine co-delivery had on the immune responses in these mice.

Overall, intradermal DNA immunization with larger doses of pcDNA3.1zeo^{+} containing the heterologous, but not the homologous TSHR was an effective method to induce hyperthyroidism in a small proportion (37.5 %) of BALB/cJ mice. Surprisingly, this was not enhanced through co-delivery of cytokine-vectors. These results confirm that hTSHR-specific autoantibodies produced in these mice can recognize determinants of the murine TSHR that stimulate the thyrocytes and cause thyroid hyperactivity. The lack of disease induction in all antibody-positive mice most likely reflects the delicate balance between different TSHR autoantibodies *in vivo* (i.e. TSAb, TSBAb, and neutral TSHR Ab), an obstacle that will be difficult to overcome in future experiments when trying to induce hyperthyroidism in 100% of the mice.

CHAPTER FOUR

AN ALGORITHM-BASED APPROACH FOR MAPPING A^K-BINDING IMMUNOGENIC TSHR EPITOPES

4.1 ABSTRACT

Autoantibodies specific for the TSHR have a major aetiological role in Graves' disease. However, very little is known about the T cells that regulate TSHR autoantibody production or what epitopes they recognize. Using an algorithm-based approach, we identified 3 hTSHR peptides with A^k-binding potential. Two of these peptides, hTSHR438-449 and hTSHR716-731, corresponding to regions of the transmembrane and intracellular domains of the hTSHR molecule respectively, were synthesized and their immunogenicity and pathogenicity was tested in AKR/J ($H\text{-}2^k$) mice. *In vivo* priming with either peptide led to moderate proliferative secondary LNC responses to each peptide *in vitro* and low levels of IL-2 production. Peptide-specific serum IgG Abs were also induced following priming and boosting of mice with either peptide, confirming their immunogenicity. hTSHR438-449-specific proliferation was inhibited (~ 50%) in the presence of both anti-A^k and anti-E^k mAb confirming the predictive value of the algorithm used for identifying A^k-binding peptides. Peptide immunogenicity, however, did not correlate with pathogenicity, since neither peptide caused mononuclear cell infiltration of the thyroid. This lack of correlation may be due to the heterologous nature of the peptides or may be characteristic of the mouse strain used. Immunization of additional $H\text{-}2^k$ mouse strains, and adoptive transfer experiments should be carried out before the pathogenic potential of these peptides is ruled out.

4.2 INTRODUCTION

Although it is well established that autoantibodies to the TSH receptor play a central role in the pathogenesis of GD, little is known about the T cells that regulate TSHR-specific autoantibody production. Recognition of the TSHR by T-helper (Th) cells *in vivo* must be carried out by T cell receptors (TCR) specific for TSHR peptides presented in the context of MHC class II molecules on autologous APCs. Identifying the T cell epitopes within the TSHR, the MHC molecule(s) involved in their antigen presentation, and the genes encoding TSHR-specific TCRs, would improve our understanding of the pathogenesis of autoimmune GD, and might facilitate development of specific immunosuppressive therapies.

Among the different methods employed to map T-cell epitopes in autoantigens, the use of computerized algorithms is quite common. Carayanniotis and colleagues, for example, have mapped two pathogenic Tg epitopes using both the 'AMPHI' and 'tetramer motif' algorithms (reviewed in Carayanniotis et al.[175]). Both peptides (p2494-2510 and p2694-2711) induced strong secondary proliferative responses in cultures of LNCs from EAT susceptible mice and lymphocytic infiltration in their thyroids. More recently, computer algorithms have been shown to predict peptide sequences that can bind to A^k and E^k molecules (reviewed in Nelson, et al.[176]). The computer algorithm developed by Altuvia et al.[157], for example, was created by identifying common motifs of known A^k- or E^k-binding peptides. These motifs rely on unique features of the a.a. in each position, such as polarity, charge, size, and hydrophobicity. Preliminary data have

identified 5 new pathogenic T-cell epitopes from the mouse Tg sequence using this particular algorithm (G. Carayanniotis, unpublished data).

The objective of my research was to use the same algorithm to scan the entire hTSHR molecule for peptides that may bind to A^k. Candidate peptides were then tested in AKR/J (H-2^k) mice for their capacity to bind to A^k and E^k molecules and induce T-cell and antibody responses, as well as for their ability to generate pathogenic T cells that infiltrate the thyroid gland.

4.3 RESULTS

4.3.1 Identification of potential A^k-binding hTSHR peptides

Potential A^k-binding human TSHR peptides were identified using motifs A and B (Table 2.2) from the computer algorithm described by Altuvia et al.[157]. The 764 amino acid residues of the hTSHR molecule were scanned using the Network Protein Sequence analysis website and 26 peptides that fit motif A as well as 9 peptides that fit motif B were identified (Table 4.1). Of these, 3 peptides contained partially overlapping and 4 sequences contained completely overlapping A and B motifs (Table 4.2A & B). Closer examination of these peptides revealed three distinct sequences corresponding to the extracellular, transmembrane, and intracellular domains of the native hTSHR protein. Peptides designated hTSHR79-94, hTSHR 438-449, and hTSHR716-731 (Table 4.3) were selected as sites of putative T-cell epitopes. The hTSHR79-94 was also identified when the mTSHR sequence was scanned using motif A and B (Table 4.4A & B). In addition, another A^k-peptide binding motif (D-X-X-[ILVN]-X-[EQ]-{HRK}-X-

Table 4.1 Identification of human TSHR sequence coordinates that fit A^k-binding motifs A and B from the computer algorithm described by Altuvia et al.[157]

Motif	Peptide Coordinates[a]	hTSHR Sequence
A	61-67	ETHLRTI
	86-92	DVTLQQL
	105-111	HIEIRNT
	124-130	ELPLLKF
	135-141	NTGLKMF
	178-184	ETLTLKL
	210-216	NKYLTVI
	235-241	QTSVTAL
	289-295	QKKIRGI
	360-366	EDEIIGF
	374-380	QEETLQA
	375-381	EETLQAF
	394-400	EDMVCTP
	432-438	NVFVLLI
	443-449	HYKLNVP
	506-512	ELSVYTL
	573-579	DTETPLA
	575-581	ETPLALA
	601-607	HVKIYIT
	617-623	DKDTKIA
	650-656	NKPLITV
	658-664	NSKILLV
	674-680	NPFLYAI
	686-692	QRDVFIL
	718-724	DIQVQKV
	722-728	QKVTHDM
B	41-45	CKDIQ
	82-86	YVSID
	86-90	DVTLQ
	319-323	NSPLH
	443-447	HYKLN
	474-478	DLYTH
	483-487	NHAID
	718-722	DIQVQ
	722-726	QKVTH

[a]Coordinates correspond to published a.a. positions within the mature hTSHR polypeptide including the signal sequence[25].

Table 4.2A Prediction of I-A^k-binding T-cell epitopes within the human TSHR using partially overlapping A and B motifs from Altuvia et al.[157]

Peptide Coordinates[a]	hTSHR Sequence[b]
82-92	**YVSI**<u>**D**</u>VTLQQL
718-726	<u>DIQV</u>**QKVTH**
718-728	**DIQV**<u>**Q**</u>KVTHDM

[a]Coordinates correspond to published a.a. positions within the mature hTSHR polypeptide including the signal sequence[25].
[b]Sequences denoted by motif A and B are <u>underlined</u> and represented in **bold** letters respectively.

Table 4.2B Prediction of I-A^k-binding T-cell epitopes within the human TSHR using completely overlapping A and B motifs from Altuvia et al.[157]

Peptide Coordinates[a]	hTSHR Sequence[b]
86-92	**<u>DVTLQQL</u>**
443-449	**<u>HYKLNVP</u>**
718-724	**<u>DIQVQKV</u>**
722-728	**<u>QKVTHDM</u>**

[a]Coordinates correspond to published a.a. positions within the mature hTSHR polypeptide including the signal sequence[25].
[b]Sequences denoted by motif A and B are <u>underlined</u> and represented in **bold** letters respectively.

Table 4.3 hTSHR peptides chosen as sites of putative T cell epitopes

Peptide Coordinates[a]	**hTSHR Sequence**[b]
79-94	SRI~~Y~~VS~~I~~**D**VTLQQLES
438-449	ILLTS**HYKLN**VP
716-731	ST**DIQV**(**Q**~~K~~V~~T~~H**D**M)RQG

[a]Coordinates correspond to published a.a. positions within the mature hTSHR polypeptide including the signal sequence[25].

[b]Sequences denoted by motif A are underlined or (bracketed), while sequences denoted by motif B are represented in bold or ~~strikethrough~~ letters.

Table 4.4A Prediction of I-A^k-binding T-cell epitopes within the murine TSHR using partially overlapping A and B motifs from Altuvia et al.[157]

Peptide Coordinates[a]	mTSHR Sequence[b]
82-92	**YISID**ATLQRL
120-130	**DALTE**LPLLKF

[a]Coordinates correspond to published a.a. positions within the mature mTSHR polypeptide including the signal sequence[63].
[b]Sequences denoted by motif A and B are underlined and represented in **bold** letters respectively.

Table 4.4B Prediction of I-A^k-binding T-cell epitopes within the murine TSHR using completely overlapping A and B motifs from Altuvia et al.[157]

Peptide Coordinates[a]	mTSHR Sequence[b]
86-92	**DATLQRL**
120-126	**DALTELP**

[a]Coordinates correspond to published a.a. positions within the mature mTSHR polypeptide including the signal sequence[63].
[b]Sequences denoted by motif A and B are underlined and represented in **bold** letters respectively.

[STRQAG]) published by Fremont et al.[177] derived from features of the crystal structure of A^k also identified the hTSHR sequence (hTSHR86-94) that corresponded to part of the hTSHR79-94 peptide. The fact that 2 different algorithms identified the same region of the hTSHR as a potential A^k-binding peptide, and that scanning the mTSHR sequence identified the same sequence coordinates that fits motif A and B as the hTSHR sequence strongly suggests the potential importance of this peptide. Unfortunately, this peptide could not be synthesized due to technical problems in its quality control and was not included in this study. The hTSHR438-449 peptide, on the other hand is 92% homologous to the corresponding murine sequence[63], differing by only 1 amino acid at position 447 (N → T) (Figure 4.1A). Unlike the hTSHR438-449 peptide sequence, which contains completely overlapping A and B motifs (Table 4.3), the corresponding mTSHR sequence only contains a sequence that fit motif A (mTSHR443-449) (data not shown). In contrast, the hTSHR716-731 peptide is only 56% homologous to its mouse counterpart (Figure 4.1B), and despite the existence of 4 different sequences that fit motif A and B within the 16-mer peptide (Table 4.3), the corresponding murine sequence lacks the A or B motifs (data not shown).

4.3.2 Immunogencity of hTSHR peptides

To determine the immunogenicity of the hTSHR peptides, AKR/J mice (H-2^k) were challenged s. c. with 200 nmol of hTSHR438-449 or hTSHR716-731, and 9 days later, peptide-specific proliferative LNC responses were determined. As shown in Figure 4.2, LNC from AKR/J mice proliferated moderately to both hTSHR438-449 and hTSHR716-731 peptides *in vitro*, exhibiting stimulation index (S.I.) values ≥ 2 when

(A)

Human	**(438-449)**	I L L T S H Y K L N V P
Murine	**(438-449)**	- - - - - - - - - - T - -

(B)

Human	**(716-731)**	S T D I Q V Q K V T H D M R Q G
Murine	**(716-731)**	- - G - - I - - I P Q - T - - S

Figure 4.1 Primary a.a. sequence of hTSHR438-449 (A) and hTSHR716-731 (B) peptides and homologies with its murine counterpart. hTSHR438-449 and hTSHR716-731 peptide sequences are 92% and 56% homologous to the corresponding murine sequence respectively. Identical a.a. between human and murine TSHR sequences are represented by a (-). Numbers indicate relative a.a. positions within the human and murine TSHR sequences.

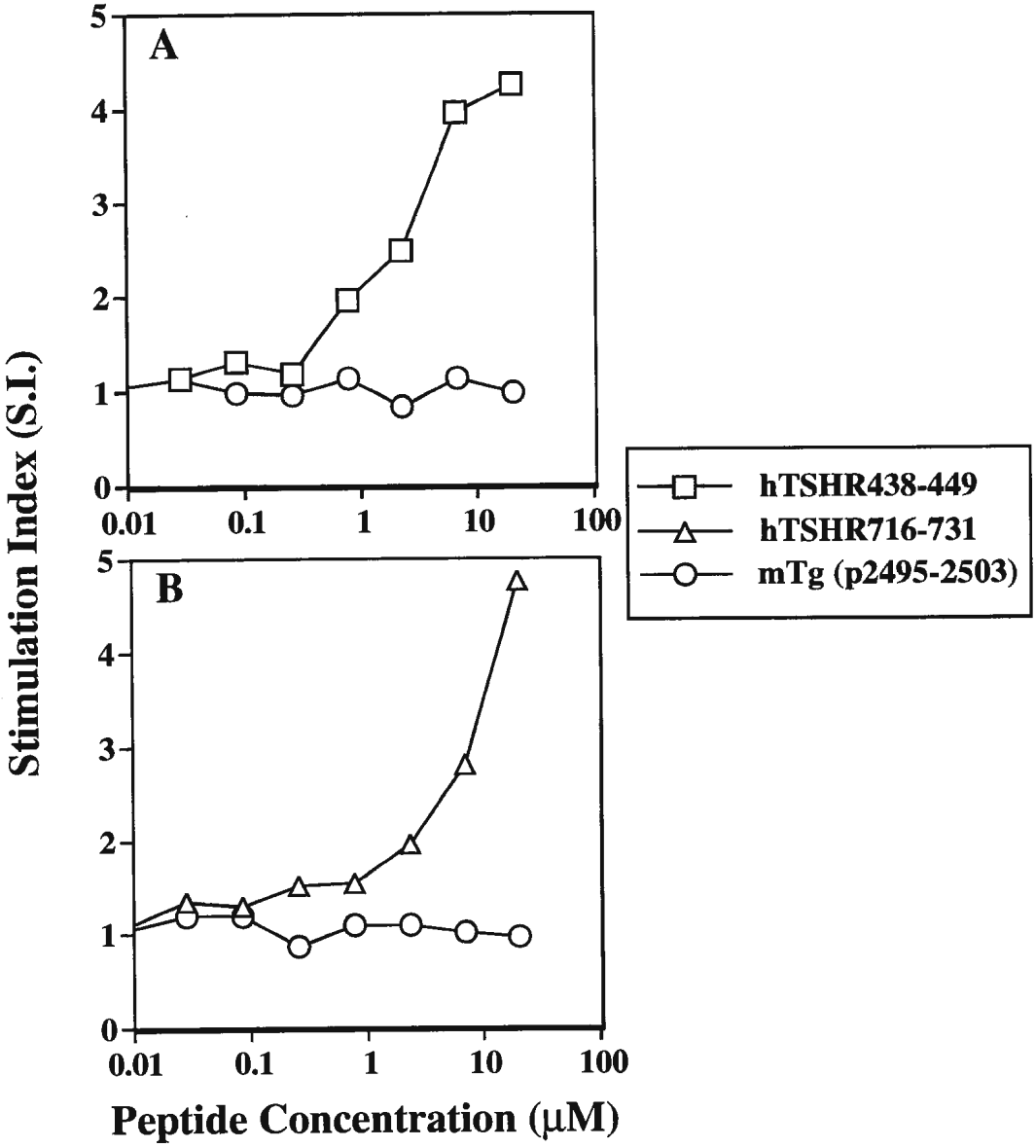


Figure 4.2 Proliferative *in vitro* responses to titrating amounts of the indicated peptides of pooled LNC from AKR/J mice (two mice per group) s.c. primed 9 days earlier with 200 nmol of either hTSHR438-449 (A) or hTSHR716-731 (B) peptide. Stimulation index is defined as CPM in the presence of antigen/CPM in the absence of antigen. Background cpms were between 963 and 1240. Murine Tg peptide p2495 (a.a. 2495-2503) was used as a non-specific peptide control. The data are representative of 2 experiments.

peptide concentrations were between 2 and 20 μM. The responses were specific since no proliferation (S.I. ≤ 2) was observed in cultures containing a control mTg peptide (a.a. 2495-2503; LINRAKAVK). To ascertain the cytokine profiles of T cells responding to hTSHR peptides, supernatants were collected from cultures of primed LNCs incubated with 15 μM of the appropriate peptide. The supernatants were assayed for IL-2, IFN-γ, IL-4, and IL-10 using a cytokine sandwich ELISA. As shown in Table 4.5, both peptides induced the production of IL-2 from specific T cells, correlating with T-cell proliferation. IL-2 production, however, did not correlate with IFN-γ production since IFN-γ levels were less than the detection limit (62.5 pg/ml, Table 4.5). IL-4 and IL-10 were also not detected in the same supernatants. These results suggest that both hTSHR peptides are weak immunogens at the T-cell level.

4.3.3 The hTSHR438-449 peptide is recognized by I-A^k and I-E^k restricted T cells in proliferative assays

To determine whether hTSHR peptides bind to I-A^k or I-E^k MHC class II molecules, a proliferation assay was performed in the presence of either anti-A^k or anti-E^k mAbs. The functionality of both monoclonal Abs was verified by their ability to inhibit the proliferative responses of I-A^k and I-E^k restricted T cell hybridomas[152] in a dose-dependent manner (data not shown). As shown in Table 4.6, hTSHR438-449-specific proliferation of primed LNCs from AKR/J mice was inhibited (~ 50%) in the presence of both anti-A^k and anti-E^k mAb. No significant blocking was observed in the presence of control mAb (HB65) specific for influenza A nucleoprotein. These data confirm the predictive value of algorithms used for identifying A^k-binding peptides. Similar

Table 4.5 Cytokine production from primed AKR/J mouse LNCs in response to hTSHR peptide *in vivo*

Peptide		Cytokine Production (pg/ml)[c]			
in vivo[a]	***in vitro***[b]	**IL-2**	**IFN-γ**	**IL-4**	**IL-10**
hTSHR438-449	**hTSHR438-449**	26.7	<62.5	<7.8	70.53
	mTg (p2495-2503)	8.7	<62.5	<7.8	79.72
hTSHR716-731	**hTSHR716-731**	17.0	<62.5	<7.8	48.68
	mTg (p2495-2503)	<7.8	<62.5	<7.8	54.72

[a]AKR/J mice were s.c. immunized with 200 nmol of the indicated peptide in CFA.

[b]LNCs obtained 9 days following priming mice were restimulated *in vitro* with 15 μM of the indicated peptide.

[c]Culture supernatants were collected 48h later and assessed for the presence of cytokines using a sandwich ELISA. Data represent mean concentration values extrapolated from ELISA standard curves. Detection limits from the cytokine ELISA were as follows: IL-2 and IL-4 = 7.8 pg/ml, IL-10 = 15.6 pg/ml, and IFN-γ = 62.5 pg/ml.

Table 4.6 Inhibition of hTSHR438-449-specific LNC proliferation by various mAb *in vitro*

Blocking mAb	***In vitro* proliferation (cpm ± SD)[a]**	**% Inhibition of Proliferation [b]**
None	6158 ± 1167	0
Anti-IA^k	2921 ± 575	53
Anti-IE^k	2812 ± 544	54
Anti- influenza A NP	5707 ± 755	7

[a]LNCs from AKR/J mice primed *in vivo* with 200 nmol of the indicated peptide in CFA and 9 days later were cultured *in vitro* with 13.3 μM of the immunizing peptide and 10 μg/ml of the indicated mAb. Background cpm did not exceed 1200.

[b]Percent inhibition of proliferation was calculated as described in Materials and Methods.

experiments were performed with the hTSHR716-731 peptide, however, complications with the TopCount NXT™ following the inhibition assay precluded data analysis.

4.3.4 hTSHR peptides induce strong IgG responses in AKR/J mice but not thyroid lymphocytic infiltration

An alkaline-phosphatase based ELISA was used to assess peptide-specific IgG in pooled sera (day 35) from 5-6 AKR/J mice primed (day 0) with hTSHR438-449 or hTSHR716-731 peptide in CFA and boosted on day 21 with the same Ag in IFA. Both peptides induced moderate levels of peptide-specific IgG after 5 weeks (Table 4.7). Thus at the serological level, both hTSHR peptides are clearly immunogenic. To determine peptide pathogenicity, thyroids from the same mice were removed and histologically examined for mononuclear cell infiltration. Despite proliferative T-cell responses (Section 4.3.2) and antibody production, no lymphocytic infiltration was observed in any of the mice from either group (Table 4.7).

4.4 DISCUSSION

Although autoantibodies specific for the TSHR are the direct cause of hyperthyroidism in GD[1], the initiation and/or the amplification of autoantibody production is largely T-cell dependent. In addition, thyroid glands of GD patients contain infiltrating activated T lymphocytes. Thus, identifying the epitopes recognized by these T cells would not only improve our understanding of the pathogenesis of autoimmune GD, but would facilitate development of specific immunosuppressive therapies. Several diverse strategies have been used to map T-cell epitopes of various autoantigens. Computerized algorithms, for example, have helped identify two pathogenic Tg epitopes by selecting

Table 4.7 EAT and serum IgG responses in AKR/J mice challenged with hTSHR peptides

Peptide[a]	Infiltration Index						Mice with EAT[b]	Serum IgG response (OD 405 nm) to[c]					
								hTSHR 438-449		hTSHR 716-731		Murine lysozyme	
	0	1	2	3	4	5		1:80	1:160	1:80	1:160	1:80	1:160
hTSHR 438-449	5	0	0	0	0	0	0/5	0.576	0.428	ND	ND	0.167	0.127
hTSHR 716-731	6	0	0	0	0	0	0/6	ND[d]	ND	0.941	0.504	0.245	0.133

[a]AKR/J mice were primed and boosted with the indicated peptide as described in Materials and Methods.
[b]Direct EAT induction was assessed by scoring thyroid mononuclear cell infiltration as described in Materials and Methods.
[c]Specific IgG was assessed by an alkaline-phosphatase based ELISA in pooled sera (collected 5 weeks from initial challenge) from each group, tested at the dilutions shown. Results are expressed as mean OD (405 nm) of triplicate wells.
[d]ND = not determined

peptides with physico-chemical and structural properties that enable their binding to MHC class II molecules[151;156;178]. Using an algorithm that predicts A^k and E^k-binding potential[157], we have identified 2 immunogenic hTSHR peptides: hTSHR438-449 and hTSHR716-731.

The 764 a.a. of the TSHR molecule is organized into three distinct regions, an extracellular domain of 398 amino acids, followed by the 264 amino acid transmembrane spanning domain, and an 82 amino acid intracellular carboxyl-terminal domain[179]. The two peptides used in this study, hTSHR438-449 and hTSHR716-731, corresponded to the transmembrane and intracellular domains respectively. Both peptides contained completely overlapping A and B motifs, and in the 16-mer (hTSHR716-731), more than one A and B motif were found (Table 4.3).

The moderate peptide-specific *in vitro* proliferative response of hTSHR438-449-primed LNCs from AKR/J mice (Figure 4.2A), was inhibited in the presence of anti-A^k monoclonal Ab (Table 4.6). These results indicate that this peptide is recognized by A^k-restricted T-cells and therefore highlight the value of this computer algorithm in identifying A^k-binding peptides. Interestingly, the E^k-specific mAb similarly inhibited hTSHR438-449-specific LNC proliferation (Table 4.6), perhaps because this peptide can also be presented in the context of I-E^k. It is not uncommon for the same peptide to bind to both I-A and I-E molecules. For example, Balasa, et al.[178], blocked peptide-specific proliferative responses of rat LNC *in vitro* with mAbs specific for RT1-B (I-A equivalent) and RT1-D (I-E equivalent) antigens. Our result, however, was not expected

since the hTSHR438-449 peptide was not identified as a potential E^k-binder by the same computer algorithm of Altuvia, et al.[157] (results not shown).

Although both hTSHR peptides yielded stimulation indices (S.I.) > 3 when primed LNCs from AKR/J mice were incubated with 15 μM of the immunizing peptide (Figure 4.2A & B), only low levels of IL-2 were detected in the culture supernatants (Table 4.5). Thus, these results do not support the induction of Th1 cells during the immunization period despite the fact that CFA is known to elicit Th1 cells.

Despite the induction of proliferative T-cell responses (Figure 4.2) and specific IgG responses (Table 4.7), neither peptide induced thyroid lymphocytic infiltration (Table 4.7). This is not surprising since proliferative LNC responses do not always correlate with peptide pathogenicity. CBA mice, for example, mount strong proliferative T-cell responses to bTg, but bTg is not pathogenic in this strain[180]. In contrast, some Tg peptides, such as rTg (2499-2507), directly elicit weak EAT but appear nonimmunogenic by proliferation assays[152]. Also, peptides that do not directly induce proliferative LNC responses or thyroid pathology are nevertheless known to mediate EAT by adoptive transfer of peptide-specific LNC into naïve hosts[181]. This protocol usually leads to consistent EAT induction and a more severe lymphocytic infiltration of the thyroid than direct challenge of mice with peptide in adjuvant. Thus, the lack of EAT induction after direct challenge of mice with either hTSHR peptides does not exclude their pathogenic potential. Unfortunately, time constraints prevented us from testing EAT induction by the adoptive transfer method.

Other factors such as the genetic environment of the host and the heterologous nature of the hTSHR peptides may have also contributed to the lack of peptide pathogenicity. Ideally, peptide screening should be simultaneously tested in mice of several MHC haplotypes. Besides the fact that the algorithm used in this experiment selects A^k-binding peptides, we chose AKR/J mice because the H-2^k haplotype is known to be susceptible to EAT induction[182]. Moreover, TSAb have been induced in AKR mice following immunization with fibroblasts stably expressing hTSHR and MHC class II molecules, which correlated with elevated T4 levels, indicating this strain is also susceptible to hyperthyroidism. Nevertheless, we cannot exclude the possibility that these peptides could be pathogenic in other H-2^k mouse strains (i.e. C3H, CBA, or BALB/K), or in mice of other MHC haplotypes. Similarly, a.a. differences between heterologous hTSHR peptide sequences and corresponding mTSHR sequence may have abolished the peptides pathogenic properties. Evidence supporting this idea was shown when human Tg peptides, highly homologous to rat pathogenic fragments were unable to induce EAT in mice[183]. Amino acid differences between homologous and heterologous proteins may also prevent the detection of pathogenic peptides by an algorithmic approach or, conversely, identify peptides that are not pathogenic.

In conclusion, the computer algorithm used in this experiment has been successful in identifying 2 immunogenic T-cell epitopes, one of which was shown to bind to A^k and E^k molecules. Further experimentation with these peptides in different mouse strains and by adoptive transfer assays is required to properly assess their pathogenicity.

CHAPTER FIVE

PROGRESS TOWARD IDENTIFICATION OF PATHOGENIC T CELL EPITOPES USING DNA IMMUNIZATION

5.1 ABSTRACT

EAT, an analogue of Hashimoto's thyroiditis in humans, is experimentally induced in mice by injection of Tg or Tg peptides emulsified in adjuvant. To date, five immunopathogenic Tg peptides have been identified. Diverse strategies have been employed to help identify other pathogenic Tg epitopes, all of which require the synthesis of peptides, which can be quite expensive. Immunization of mice with plasmid DNA encoding Tg gene segments can, in theory, induce EAT and identify larger pathogenic regions. If practical, this method would obviate the need for synthetic peptides as immunogens. Here, we designed DNA vectors encoding mTg fragments known to harbour one or several pathogenic T-cell epitopes. These vectors were used to transiently transfect APCs (TA3 and LS 102.9) that were subsequently tested for their capacity to present MHC class II/peptide complexes to appropriate peptide-specific T cell hybridomas. None of the T cell hybridomas were activated when incubated with TA3 or LS 102.9 cells transiently transfected with pcDNA3.1zeo^{+} encoding p2494 and p2694 mTg peptides, or when transfected with plasmid DNA encoding the carboxyl terminus of mTg encompassing both pathogenic peptides. T cell hybridomas were, however, activated when transfected APCs were pulsed with the appropriate exogenous peptide. These results suggested that either the synthesis of peptides in the cytosol was inefficient, or that the peptides could not be targeted to the MHC class II pathway.

5.2 INTRODUCTION

Murine EAT, an analogue of Hashimoto's thyroiditis in humans, is a T-cell-mediated autoimmune disease that can be experimentally induced in mice by injection of Tg and adjuvant[184]. The evidence that Tg-mediated EAT is under H-2 control[185] and that Tg-specific T cells are responsible for disease suggests that the large Tg molecule harbors only a limited number of thyroiditogenic peptides that can be recognized by MHC-restricted T cells. To date, five immunopathogenic Tg peptides have been identified, none of which appears to be immunodominant (reviewed in Carayanniotis et al.[175]). Epitope mapping methodologies including the use of overlapping synthetic peptides or immunoenzymatic techniques are laborious, expensive and impractical when applied to autoantigens of high molecular mass, such as Tg (homodimeric mass = 660 kDa). Alternative mapping approaches based on computer algorithms have helped in the selection of peptides with physico-chemical and structural properties that enable their binding to MHC class II molecules. Although this approach has been successful on several occasions[151;156;178], it still requires synthesis of peptides that can be quite expensive. Avoiding peptide synthesis altogether by immunizing mice with DNA encoding potential pathogenic peptides rather than the peptides themselves would not only cut the cost associated with peptide synthesis, but could identify larger pathogenic regions for further analysis. Several studies have prevented or suppressed experimental or spontaneous autoimmune disease induction in mice following immunization with DNA encoding the appropriate self-antigen[97-101]. However, it has not been tested whether vaccination with DNA encoding a self-peptide will abrogate self-tolerance and lead to an

autoimmune response. If so, there will be important implications for mapping of disease-causing T-cell epitopes in large self-proteins. This chapter describes the initial progress toward developing DNA vectors encoding murine Tg (mTg) fragments known to harbor one or several pathogenic T-cell epitopes, which will eventually be used for future mouse immunizations.

5.3 RESULTS

5.3.1 Sequencing of cDNA segments encoding murine p2494 and p2694 Tg peptides and the carboxyl terminus of murine Tg

Murine Tg cDNA segments encoding pathogenic T cell epitopes were amplified using RT-PCR and subcloned into the multicloning site of pcDNA3.1zeo^{+} mammalian expression vector as described in Materials and Methods (Section 2.5). The dideoxynucleotide chain termination method was used to sequence the cDNA inserts encoding murine p2494 (a.a. 2494-2510) and p2694 (a.a. 2694-2711) Tg peptides, and the carboxyl terminus of murine Tg. The nucleotide and derived amino acid sequence of each minigene are shown in Figure 5.1 (A, B, & C). Sequences were identical to the published mTg sequence[153] with the exception of one nucleotide substitution from thymine (T) to cytosine (C) at position 8110 of p2694 and mTg-carboxyl terminal nucleotide sequences (Figure 5B and 5C respectively). The nucleotide substitution did not result in an amino acid substitution. Artificial start and stop codons, as well as restriction enzyme sites remained intact.

5.3.2 Transfection Efficiency of TA3 and LS 102.9 APC Cells: Luciferase Assay

The transfection efficiency of both TA3 and LS 102.9 antigen-presenting cell lines was determined using the luciferase reporter gene system. Cell lysates of TA3 or

Figure 5.1 Nucleotide and derived amino acid sequences of the cDNA encoding murine p2494 (A) and p2694 (B) Tg peptides and the carboxyl terminus of mTg (C). Note the artificial start and stop codons (double underlined), as well as the *KpnI* and *XbaI* restriction enzyme sites (underlined) introduced at the 5' and 3' ends of each cDNA insert to facilitate peptide transcription and translation and subcloning into pcDNA3.1zeo^{+} mammalian expression vector respectively. Sequences were identical to the published murine thyroglobulin sequence with the exception of one nucleotide substitution from thymine (T) to cytosine (C) at position 8110 (*) of p2694 and mTg-carboxyl terminus nucleotide sequences. Sequence coordinates correspond to the published mTg sequence without the leader sequence.

(A) p2494

```
                    7480
                     |
5'ggt acc gcc atg ggg ctt atc aat agg gca aag gct gtg aag caa ttt gaa gag agc caa
                    G   L   I   N   R   A   K   A   V   K   Q   F   E   E   S   Q

  7530
   |
ggc tga tct aga 3'
 G
```

(B) p2694

```
                    7480
                     |
                                                                *
5'ggt acc gcc atg tgc tcc ttc tgg tcc aag tac atc cag act ctg aag gat gca gat gga
                    C   S   F   W   S   K   Y   I   Q   T   L   K   D   A   D   G

                7530
                 |
gcc aag gat gca tga tct aga 3'
 A   K   D   A
```

(C) mTg-carboxyl terminus

```
                    7480
                     |
5'ggt acc gcc atg ggg ctt atc aat agg gca aag gct gtg aag caa ttt gaa gag agc caa
                    G   L   I   N   R   A   K   A   V   K   Q   F   E   E   S   Q
ggc cga acc aac agc aaa aca gcc ttt tac cag gca ctg cag aat tca ctc ggt ggt gag
 G   R   T   N   S   K   T   A   F   Y   Q   A   L   Q   N   S   L   G   G   E
gac tca gat gcc cgc atc ctt gct gct gct gta tgg tat tac tcc ttg gag cac tcc aca
 D   S   D   A   R   I   L   A   A   A   V   W   Y   Y   S   L   E   H   S   T
gac gac tat gcc tcc ttc tct cgg gca ctg gag aat gcc acc cgg gac tac ttt atc atc
 D   D   Y   A   S   F   S   R   A   L   E   N   A   T   R   D   Y   F   I   I
tgt ccc atg gtc aac atg gcc agc ctc tgg gca agg aga acc cgt gga aat gtc ttc atg
 C   P   M   V   N   M   A   S   L   W   A   R   R   T   R   G   N   V   F   M
tac cat gtc ccc gaa agc tat ggc cat ggc agc cta gaa ttg ctg gca gat gtt caa tat
 Y   H   V   P   E   S   Y   G   H   G   S   L   E   L   L   A   D   V   Q   Y
gct ttt gga ctg ccc ttc tac tct gcc tac cag ggc cag ttt tca acg gag gag cag agc
 A   F   G   L   P   F   Y   S   A   Y   Q   G   Q   F   S   T   E   E   Q   S
ctg tcc ctg aaa gtc atg cag tat ttc tcc aac ttc atc aga tca gga aat cct aac tac
 L   S   L   K   V   M   Q   Y   F   S   N   F   I   R   S   G   N   P   N   Y
cca cat gag ttc tca agg aag gca gct gag ttt gcc aca cct tgg cca gac ttt att cct
 P   H   E   F   S   R   K   A   A   E   F   A   T   P   W   P   D   F   I   P
gga gct gga gga gag agc tac aag gag ctc agc gcc cag ctt ccc aat cgg cag ggc ctc
 G   A   G   G   E   S   Y   K   E   L   S   A   Q   L   P   N   R   Q   G   L
                                                        *
aaa caa gct gac tgc tcc ttc tgg tcc aag tac atc cag act ctg aag gat gca gat gga
 K   Q   A   D   C   S   F   W   S   K   Y   I   Q   T   L   K   D   A   D   G
gcc aag gat gca cag tta acc aag agt gaa gag gaa gac ttg gaa gtt gga cct gga tta
 A   K   D   A   Q   L   T   K   S   E   E   E   D   L   E   V   G   P   G   L
gaa gaa gac ctc tca ggc tca ctg gag cct gtc ccc aag agc tac agc aaa tga tga tct
 E   E   D   L   S   G   S   L   E   P   V   P   K   S   Y   S   K   Z       |
aga 3'                                                                      8241
```

LS 102.9 cells transiently transfected with varying amounts of either pGL3-Basic or pGL3-Control vectors were mixed with luciferase assay reagent (substrate) and light intensity was measured using a luminometer. As shown in Figure 5.2 (A & B), cell lysates from both cell lines transiently transfected pGL3-Control, but not pGL3-Basic vectors contain the luciferase product based on its ability to produce light following the addition of luciferin substrate. The inability of pGL3-Basic transfected APCs to produce light following substrate addition was expected due to the lack of eukaryotic promoter and enhancer sequences that would allow luciferase expression in mammalian cells. A steady increase in light production (RLU) following the addition of substrate to TA3 cell lysates previously transfected with pGL3-Control was observed and corresponded to the increasing amounts of DNA used to transfect the cells. These results confirm that TA3 and LS 102.9 cells can be efficiently transfected with plasmid DNA.

5.3.3 T Cell Activation Assay: Transiently transfected APC are unable to present peptide and activate peptide-specific T cell hybridomas

The TA3 (I-$A^{k/d}$; I-$E^{k/d}$) or LS 102.9 (H-$2A^{d/s}$ and H-E^{d}) APCs were transiently transfected with pcDNA3.1zeo^{+} constructs containing cDNA inserts encoding the pathogenic T cell epitopes p2494 (a.a. 2494-2510) or p2694 (a.a. 2694-2711). The transfected cells were then examined for their capacity to synthesize and present these peptides in the context of I-A^{k}, I-E^{k}, or I-A^{s} MHC class II molecules and activate appropriate peptide-specific T cell hybridomas. As shown in Figure 5.3 (A & B), all four T-cell hybridomas (4A2, 4A12, 5E8, and 6E10) were activated and produced IL-2 when incubated with non-transfected APCs (TA3 or LS 102.9) pulsed with their respective peptide. 4A2, 4A12, and 5E8 T cells are I-E^{k}, I-A^{k}, and I-A^{s}-restricted T cell hybridomas

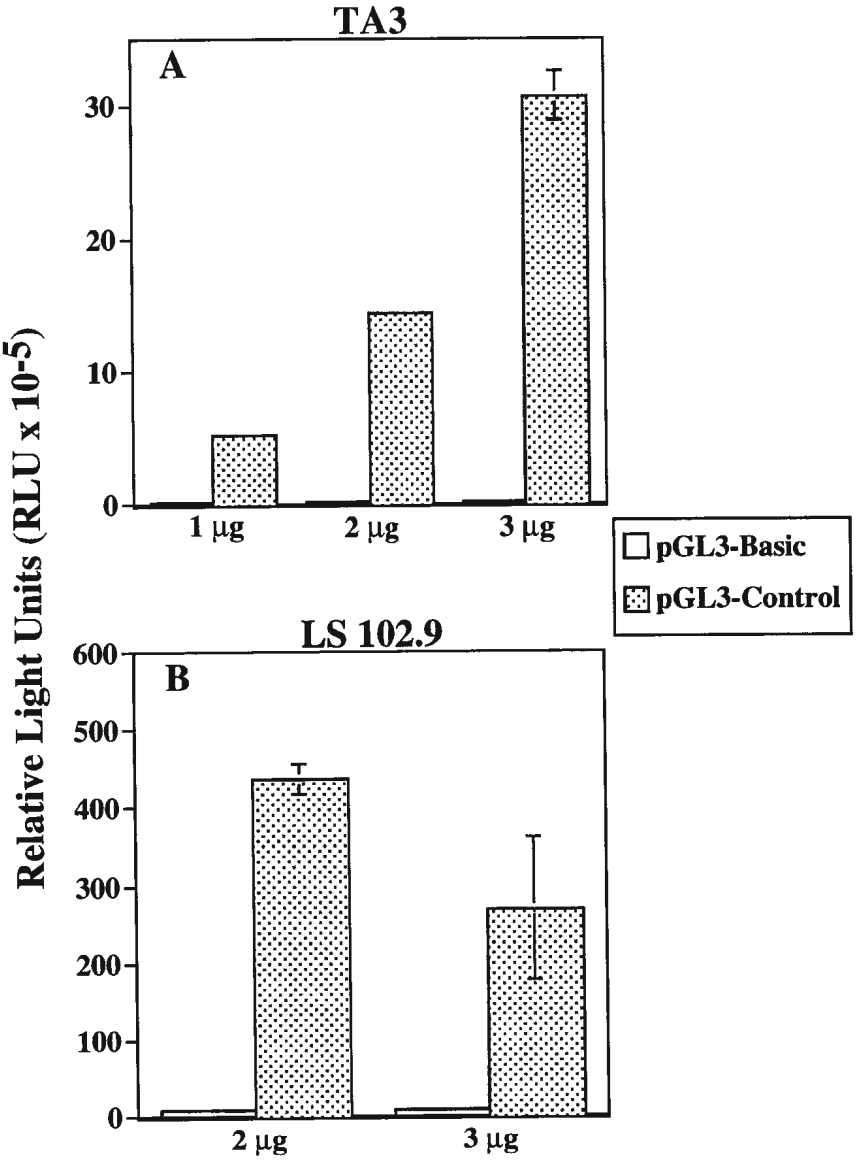


Figure 5.2 Luciferase gene expression in TA3 (A) and LS 102.9 (B) cells. Cell lysates (20 µl) from TA3 and LS 102.9 cells transiently transfected with either pGL3-Basic or pGL3-Control vectors (1, 2, or 3 µg) were mixed with luciferin substrate, and light produced following the reaction between luciferase and luciferin was measured using a Monolight® 2010 luminometer. Results are expressed as means of duplicate ± SD.

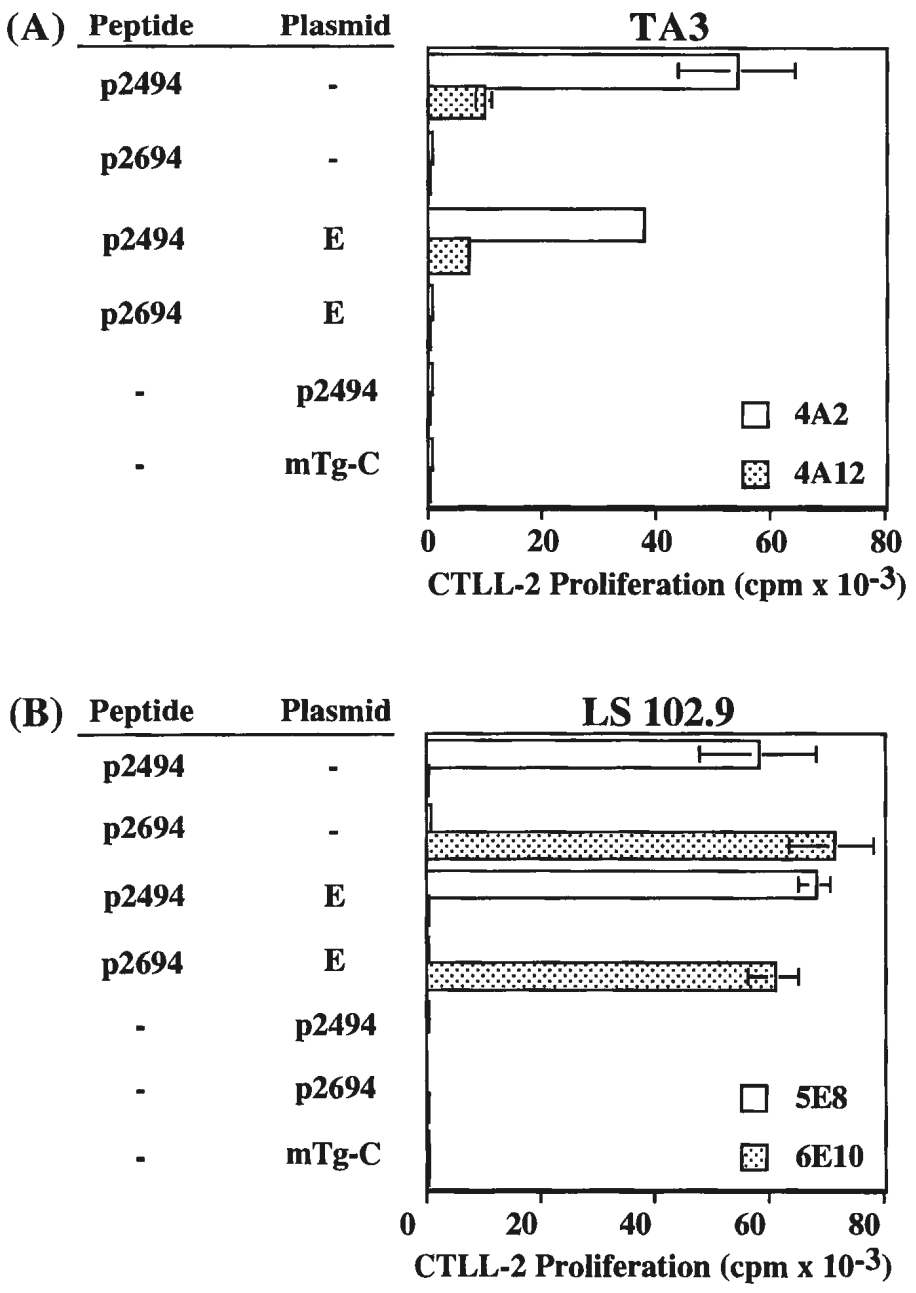


Figure 5.3 Transfected APCs are unable to activate peptide-specific T-cell hybridomas. TA3 (A) and LS 102.9 (B) cells (10^5) transiently transfected with pcDNA3.1zeo$^+$ containing minigenes encoding murine Tg peptides (p2494 and p2694) or the carboxyl-terminus of mTg were incubated with 10^5 peptide-specific T-cell hybridomas (4A2, 4A12, 5E8, and 6E10). After 24 h, supernatants (100 µl) were tested for IL-2 production using a CTLL-2 bioassay (Section 2.9.4). Untransfected APCs and APCs transfected with pcDNA3.1zeo$^+$ alone pulsed with specific and non-specific peptide were used as controls. Results are expressed as means of triplicates ± SD.

respectively, specific for p2494, whereas 6E10 is an I-A^s-restricted T cell hybridoma specific for p2694 peptide. Activation was not observed after pulsing of APC with the control peptide (p2694 for 4A2, 4A12, and 5E8; p2494 for 6E10). The antigen presenting machinery of both TA3 and LS 102.9 cells remained intact following the 48-hour transfection period, since APCs transfected with the empty vector (pcDNA3.1zeo$^+$) and pulsed with exogenous peptide could activate the T cell hybridomas. None of the T cell hybridomas were activated when incubated with TA3 or LS 102.9 cells transiently transfected with pcDNA3.1zeo$^+$ encoding p2494 and p2694 peptides, or when transfected with plasmid DNA encoding the carboxyl terminus of mTg encompassing both pathogenic peptides. These results suggested that either the synthesis of peptides in the cytosol was inefficient, or that the peptides could not be targeted to the MHC class II pathway.

5.4 DISCUSSION

DNA vaccination with a plasmid encoding dominant pathogenic peptides from various autoantigens including myelin and proteolipid proteins, and the β-chain of insulin have been shown to suppress and/or prevent the onset of spontaneous and experimentally induced autoimmune diseases[97-101]. No studies, however, have attempted to abrogate self-tolerance and experimentally generate autoimmune diseases using DNA immunization. If this novel approach is able to induce disease analogous to the disease induced using conventional immunization regimes, it could have profound implications in the mapping of disease-causing T-cell epitopes of large self-proteins. We have taken the initial steps

toward developing DNA vectors encoding known EAT-inducing T-cell epitopes in the hope to induce thyroiditis in mice following DNA immunization.

Despite complete identity between the published mTg sequence[153] and cDNA insert sequences (Figure 5.1A, B, & C), as well as the good transfection efficiency of both APCs (Figure 5.2A & B), transiently transfected TA3 and LS 102.9 cells were unable to present MHC class II/peptide complexes to activate peptide-specific T cell hybridomas (Figure 5.3A & B). It is well established that the immune system uses two different antigen-presenting pathways to process exogenous and endogenous proteins. Exogenous antigens are processed in the endocytic pathway and presented on the membrane with class II MHC molecules, while endogenous antigens are processed in the cytosolic pathway and presented on the membrane with class I MHC molecules[167]. It remains unclear, however, how antigen encoded by plasmid DNA is processed and presented through the MHC class II pathway following DNA immunization. Evidence suggests that soluble protein is released from transfected cells (i.e. APC or somatic cells) and is taken up by professional APCs, which process and present peptide similar to other exogenous proteins (reviewed by Cohen et al.[102]). Our DNA vector lacked a leader or signal sequence that would allow secretion of the translated peptide from the transfected cell, and therefore the peptide probably remained in the cytosol. Recent studies indicate that some APC can introduce endogenous antigen directly into the MHC class II pathway without the protein first having to be released in soluble form[186-190]. In most instances, this process applies only to proteins with natural access to the endoplasmic reticulum (i.e. cell membrane proteins). A study performed by Brooks et al.[187] demonstrated that class

II-positive B-lymphoma cells (M12.C3) transfected with plasmid DNA encoding hen egg lysozyme (HEL) peptide (a.a. 46-61) could form HEL(46-61)/I-A^k complexes and activate a peptide-specific T-cell hybridoma (3A9) provided the carboxyl-terminal sequence of the HEL minigene included KDEL residues or, alternatively, the complete HEL-coding sequence was joined in frame with H-$2K^b$ cDNA. The KDEL sequence is a retention sequence that allows the retrieval of soluble proteins from salvage compartments into the endoplasmic reticulum of transfected cells[191]. The H-$2K^b$ cDNA, on the other hand, encodes the transmembrane and cytoplasmic domains of the MHC class I molecule promoting chimeric membrane expression of the HEL peptide. Designing plasmid vectors encoding pathogenic mTg epitopes together with sequences that promote peptide translocation to the ER or membrane would be another approach that could be used to encourage peptide MHC class II processing and presentation that may lead to peptide-specific T-cell hybridoma activation.

Secretion or translocation of peptide to the ER or cell membrane may have yielded positive results; however, we cannot exclude the possibility that the inability of both transiently transfected APCs to activate peptide-specific T cell hybridomas was caused by the lack of transcription and translation of the cDNA insert. Transcription may have been detected by RT-PCR using mRNA isolated from transfected cells and primers specific for the mTg minigenes. This technique used by Ruiz, et al.[99], however, does not confirm peptide translation. Alternatively, both transcription and translation of the cDNA inserts may be confirmed either by immunoblotting cell lysates from transfected cells using peptide-specific mAb or i*n vitro* translation. The small size of the translated

peptides would still make detection difficult using either approach. Moreover, even if these methods were able to confirm peptide expression, they do not provide any information regarding peptide presentation, in particular MHC class II presentation.

None of the studies that claimed to suppress or prevent experimental or spontaneous autoimmune disease induction when immunizing mice with DNA encoding immunodominant self-peptides confirmed peptide expression on APC before injecting mice with DNA[97-101]. Ruiz, et al.[99], for example, immunized SJL/J mice with plasmid DNA encoding the encephalitogenic residues of myelin proteolipid protein ($PLP_{139-151}$) and observed a delayed onset of disease, reduced mean peak disease severity, and reduced mean disease score compared to control mice following subsequent induction of EAE with $PLP_{139-151}$ and CFA. In addition, proliferative responses and production of Th1 cytokines (IL-2 and IFN-γ) were reduced in LNCs taken from mice injected with DNA encoding for the $PLP_{139-151}$ peptide when restimulated *in vitro*. Similar suppression of EAE clinical signs and reduced IFN-γ production were found by another group that immunized Lewis rats with DNA encoding a different encephalitogenic T cell epitope, guinea pig myelin basic protein peptide 68-85 (pZZ/MBP_{68-85})[98]. However, unlike Ruiz, et al.[99], proliferative responses to MBP_{68-85} of T cells derived from the regional lymph nodes of pZZ/MBP_{68-85}-treated rats was not significantly different from that of controls. Another study was able to reduce the incidence of insulin-dependent diabetes mellitus (IDDM) in the well-established transgenic RIP-LCMV-NP 25-3 H-2^d mouse line if the mice were immunized with plasmid expressing the porcine insulin B chain after, but not before disease induction[97]. Reduction in the incidence of IDDM was also observed in

NOD mice following i.m. injection with plasmid encoding insulin B peptide (9-23), which appeared to be mediated by insulin B (9-23)-specific down-regulation of IFN-γ production by T cells. Suggested explanations for the protection afforded by DNA vaccination include the generation of antigen-specific regulatory cells[97;170] and induction of anergy in autoreactive T cells[99]. Exactly how these regulatory cells were induced or how autoreactive T cells were anergized remains unclear. The lack of evidence supporting peptide expression further complicates the interpretation of these results. Ruiz et al.[99] did detect low titers of $PLP_{139-151}$ peptide-specific antibodies in the sera of mice immunized with DNA encoding $PLP_{139-151}$ peptide suggesting that the peptide was expressed and specific serological immune responses against that peptide occurred. However a non-specific peptide control was not used, nor were sera from mice immunized with the empty vector tested. Thus it cannot be concluded that the humoral immune response observed in these mice was peptide-specific since it might have resulted from the immunization regime itself.

Confirmation of peptide expression on APC *in vitro* prior to the initiation of *in vivo* immunization is a parameter that we feel should not be overlooked. Once expression of p2494 and p2694 is confirmed in our model, we will proceed to immunize EAT-susceptible murine strains (SJL, C3H, and B10.BR). It will be interesting to see whether EAT can be induced following DNA immunization encoding self-peptide or, like previous studies, ameliorates or prevents disease induction.

CHAPTER SIX

FUTURE DIRECTIONS

6.1 FUTURE EFFORTS TOWARD ESTABLISHING A MOUSE MODEL FOR GRAVES' HYPERTHYROIDISM

Before a mouse model of GD is deemed successful, a number of criteria must first be met. For instance, the experimental protocol should, reproducibly, induce most of the clinical signs and symptoms of GD including i) elevated thyroid hormone and/or reduced TSH; ii) TSAb activity; iii) changes in thyroid architecture; and iv) lymphocytic thyroiditis in experimental mice. Using intradermal DNA immunization with pcDNA3.1zeo^{+}/hTSHR, we were able to induce 2 of the 4 criteria (i.e. elevated TT4 and goiter) in a small proportion (37.5%) of BALB/cJ mice (Chapter 3). Unfortunately, elevated TT4 levels and enlarged thyroids in these hyperthyroid mice did not correlate with TSAb activity or lymphocytic thyroiditis. As discussed in Section 3.4, TSBAb may have masked TSAb activity *in vitro*. Before this assumption can be made, however, TSBAb activity must be confirmed. This could be accomplished by seeing whether the thyrotoxic mouse serum inhibits bTSH-induced cAMP response in JP09 cells. Alternatively, use of a different cell line (i.e. JP26) expressing comparable numbers of TSHR molecules to normal thyroid epithelial cells may have increased the sensitivity of this assay to detect TSAb by decreasing the nonspecific stimulation by normal sera.

Although most protocols aimed at establishing an animal model of Graves' hyperthyroidism induced TSHR-specific autoantibodies, very few, including ours, were able to induce lymphocytic thyroiditis. Costagliola et al.[83], however were able to induce severe lymphocytic infiltration in 14 BALB/c mice following i.m. immunization with

hTSHR cDNA. Notably, these thyroids were collected 6 and sometimes 8 weeks later than our thyroid collection time points (10 or 12 weeks postpriming). Thus, it is possible that lymphocytic thyroiditis may have developed in our hyperthyroid mice and/or mice positive for TSHR-specific Abs at a later time point.

With the exception of Kaithamana et al.[80] who was able to induce hyperthyroidism in almost 100% of experimental mice, we and others have not been able to exceed 40% disease induction regardless of TSHR-specific Ab production. As discussed in Section 3.4, this inability to induce 100% hyperthyroidism most likely reflects the delicate balance between different TSHR autoantibodies *in vivo* (i.e. TSAb, TSBAb, and neutral TSHR Ab), an obstacle that will be difficult to overcome in future studies. Isolating TSAb from the heterogeneous TSHR Ab population or generating a B cell hybridoma that secretes monoclonal TSAbs would greatly enhance our understanding of the interaction between the TSHR and TSAbs. Currently, only mAbs specific for linear[147] or conformational TSHR epitopes exist[83], all of which lack stimulatory activity. Thus, future research should aim toward generating thyroid stimulating mAbs.

6.2 ASSESSMENT OF hTSHR PEPTIDE PATHOGENICITY

In Chapter 4, an algorithm-based approach was described that helped identify 2 immunogenic hTSHR peptides (i.e. induced peptide-specific proliferative secondary LNC responses *in vitro* and specific serum IgG Abs following *in vivo* priming of AKR/J mice) that lacked pathogenicity (i.e. unable to induce thyroid lymphocytic infiltration). As discussed in Section 4.4, it is not uncommon for peptides to be immunogenic, but not pathogenic[180]. However, before this assumption can be made for our peptides, they need

to be simultaneously tested in other H-2^k mouse strains (i.e. C3H, CBA, or BALB/ K), or in mice of other MHC haplotypes. In addition, adoptive transfer experiments using peptide-specific LNC should be carried out before the pathogenic potential of these peptides is ruled out since this protocol usually leads to consistent EAT induction and more severe lymphocytic infiltration than direct challenge of mice with peptide and adjuvant.

The lack of correlation between hTSHR peptide immunogenicity and pathogenicity may also be due to the heterologous nature of the peptides. Amino acid differences between hTSHR peptide sequences and corresponding mTSHR sequences may have abolished its pathogenic properties similar to that observed with human Tg peptides and highly homologous pathogenic rat peptides[183]. For this reason, murine TSHR peptides with A^k-binding potential (4.4A & B) should also be synthesized and tested for immunogenic and pathogenic potential in various H-2^k mouse strains.

Finally, additional attempts to synthesize the hTSHR peptide corresponding to residues 79-94 should be made since 2 different algorithms[157;177] identified the same region of the hTSHR as a potential A^k-binding peptide, and that scanning the mTSHR sequence identified the same sequence coordinates (Table 4.4A & B).

6.3 REENGINEERING PLASMID MINIGENES MAY PROMOTE MHC CLASS II PROCESSING AND PRESENTATION

In Chapter 5 the initial progress toward identifying pathogenic mTg T cell epitopes using genetic immunization was described. DNA vectors encoding mTg fragments known to harbour one or several pathogenic T-cell epitopes were designed and used to transiently transfect APCs that were subsequently tested for their capacity to

present MHC class II/peptide complexes and activate peptide-specific T cell hybridomas. The lack of transcription and translation of the cDNA insert, or the inability of APCs to process and present endogenously produced Ag through the endocytic (MHC class II) pathway may have contributed to the lack of T cell activation. As discussed in Section 5.4, reengineering the cDNA insert that will allow the mTg peptide to be secreted (i.e. introduction of an IgG signal sequence) may promote classical MHC class II processing and presentation of exogenous Ag that will ultimately lead to T cell activation. Alternatively, redesigning plasmid vectors to include sequences that promote peptide translocation to the endoplasmic reticulum (ER) or cell membrane may also result in expression of mTg peptide/MHC class II complexes. This idea stems from the numerous studies[192-197] that demonstrated nonclassical MHC class II presentation of peptides derived from endogenous proteins that have natural access to the ER (i.e. cell membrane proteins). A study performed by Brooks et al.[187], for example demonstrated that the addition of KDEL residues to the carboxyl-terminus of a minigene encoding the hen egg lysozyme peptide (a.a. 46-61) leads to retention of this peptide in the ER of B-lymphoma cells (M12.C3) transfected with this vector. The same study designed a plasmid construct that linked the complete HEL coding sequence to a portion of the H-2K^b cDNA, which, following transfection of M12.C3 cells, resulted in chimeric membrane expression of the HEL peptide. In both cases, M12.C3 cells were able to activate I-A^k-restricted, HEL(46-61)-specific T cell hybridomas. It is hoped through reengineering our plasmid vectors using one or all of these strategies, we can confirm peptide expression and proceed to immunize EAT susceptible mouse strains.

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