GENERATION OF MOUSE GRAVES' OPHTHALMOPATHY MODEL WITH FULL LENGTH TSH RECEPTOR PLASMID AND CYTOKINE EVALUATION BY REAL-TIME PCR

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Contents

List of Abbreviations

γIFN	Gamma interferon
APC	Antigen presenting cell
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3', 5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
FACS	Fluorescence-activated cell sorter
FRET	Fluorescence resonance energy transfer
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GAG	Glycosaminoglycans
GD	Graves' disease
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Graves' ophthalmopathy
GPCR	G protein-coupled receptor
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
IL2	Interleukin 2
IL3	Interleukin 3
IL4	Interleukin 4

IL5	Interleukin 5
IL10	Interleukin 10
KRH	Krebs-Ringer HEPES
MHC	Major histocompatibility complex
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Q	Quencher
R	Reporter
Rn	Reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription Polymerase chain reaction
SD	Standard deviation
T_4	Thyroxine
T_3	Triiodothyronine
TBII	Thyrotropin binding inhibitor immunoglobulin
Tc	Cytotoxic T
TcR	T cell receptor
Tg	Thyroglobulin
TGF β	Transforming growth factor beta
Th	Helper T
TNF α	Tumour necrosis factor alpha
TNF β	Tumour necrosis factor beta
TPO	Thyroid peroxidase
TRAB	Anti-Thyrotropin receptor autoantibodies

TRH	Thyrotropin releasing hormone
TSAB	Thyroid stimulating antibodies
TSBAB	Thyroid-stimulation blocking antibodies
TSH	Thyrotropin
TSHR	Thyrotropin receptor

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Summary

Graves' ophthalmopathy is a potentially disfiguring, sight-threatening and frequent complication of Graves' disease. There is currently no option of preventive treatment and management consists mainly of amelioration of inflammatory processes which are usually well underway once clinical presentations become overt. Lymphocytic infiltration of muscular and connective tissues of the retroorbital space is a histological hallmark of Graves' ophthalmopathy. The pathogenesis of Graves' ophthalmopathy and whether it is the result of a Th1 or Th2 regulation remains controversial. Study of inflammatory processes and cytokine profiling in human tissue samples were limited by sample, genetic and technique heterogeneity. Therefore, it is the aim of this study, to investigate the spectrum of T-lymphocyte cytokines expressed in tissues (spleen, thyroid & orbit) of genetically immunized inbred Balb/c and outbred Swiss mice by means of Real-Time PCR. These 2 mouse strains were injected with plasmid encoding the thyrotropin receptor gene. The results showed genetic immunization worked better in Swiss outbred than Balb/c. It produced significantly higher numbers of mice positive for thyrotropin receptor autoantibody (TRAB) detection by Flow Cytometry (FACS) and Indirect competitive (TBII) assays in Swiss outbred compared to Balb/c. The titers of these 2 assays were also significantly higher in outbred than in inbred mice. γIFN was found to be more abundant in the thyroids of thyrotropin receptor vaccinated Balb/c mice than those of controls. There was a dominance of γIFN and IL2 to IL5 in the ratio calculation of the thyroidal cytokines. Thyroid-stimulation blocking antibody (TSBAB) also had a linear relationship with the expression of Th1 cytokines i.e. γIFN in the spleens and

orbits and IL2 in the orbits of Balb/c mice. Expression of Th2 cytokine IL5 was higher in Swiss outbred mice injected with thyrotropin receptor compared to controls in the splenic and thyroidal tissues. There was also a drop in expression of IL2 (Th1) cytokine in the vaccinated thyroid relative to control, which differ significantly from that in Balb/c mice. There was also a large dominance of IL5 to IL2 or γIFN expression in the ratio calculation and this contrast sharply with the findings in Balb/c mice. The cytokine profile evaluation in the orbital tissues showed down regulation of IL5 in Balb/c and γIFN, IL4 and IL5 in Swiss outbred mice. This would imply a relatively quiescent immunological environment in this tissue compartment and thus dominance of either Th1 or Th2 response cannot be determined with confidence. In this study, genetic immunization of Balb/c tended towards a Th1 bias while Swiss outbred mice tended towards a Th2 bias upon genetic immunization with the human TSHR. The 2 mouse strains were identical in the treatment, housing and maintenance. The only variance is the genetic makeup of outbred and inbred mice. Given the stronger antibody response in the Swiss outbred mice, it is possible that the genetic diversity in outbred mice contribute to a more plausible model for human Graves' disease.

I. INTRODUCTION

The thyroid gland is a butterfly shaped organ located immediately below the larynx anterior to the trachea. It secretes two important hormones, thyroxine (T_4) and triiodothyronine (T_3). These hormones cause an increase in nuclear transcription of large numbers of genes in virtually all cells of the body with resultant effect of large increases in protein enzymes, structural proteins, transport proteins, and other substances. The outcome of all these changes is a generalized increased in functional activity throughout the body and a rise in the metabolic rate. Under normal physiological condition, production of these two hormones from the thyroid gland is tightly regulated by thyrotropin (TSH) from the pituitary gland via a negative feedback loop by the secreted thyroid hormone. The hypothalamus also exerts influence on the pituitary gland via the secretion of thyrotropin releasing hormone (TRH) (Figure 1).

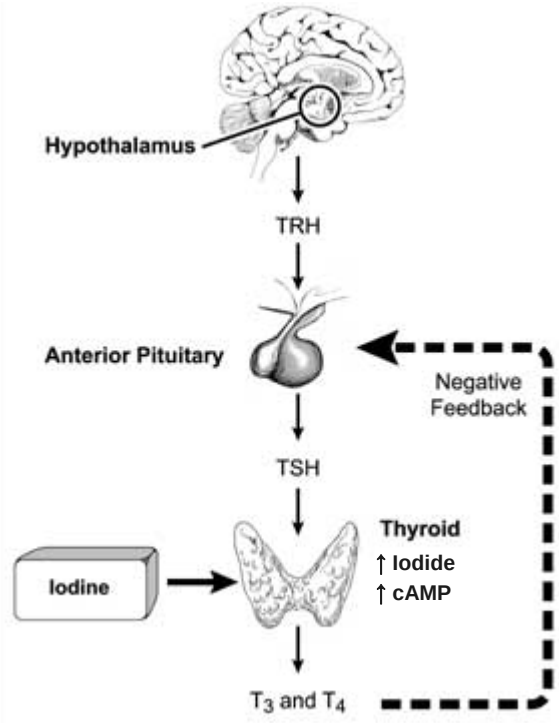


Figure 1. An illustration of the physiologic control of thyroid function.

In response to thyrotropin-releasing hormone (TRH), the pituitary gland secretes thyrotropin (TSH) which stimulates iodine trapping and increasing cAMP, thus thyroid hormone synthesis, and release of T3 and T4 by the thyroid gland. TSH is regulated by feedback from circulating unbound thyroid hormones.

1. Graves' Disease (GD)

Thyrotoxicosis is a clinical syndrome resulting from high levels of circulating thyroid hormones which increases the body's basal metabolic rate 60 - 100 per cent above the normal. This is often due to excessive thyroid secretion. Common manifestations include palpitation – sinus tachycardia or atrial fibrillation, agitation and tremor, generalized muscle weakness, proximal myopathy, angina and heart failure, fatigue, hyperkinesias, diarrhea, excessive sweating, intolerance to heat, oligomenorrhea and subfertility. There is often weight loss despite normal appetite.

By far, Graves' disease (GD) is the most common form of thyrotoxicosis and may occur at any age, with a peak incidence in the 20- to 40-year age group with a predisposition toward the female sex. Graves' disease is characterized by a generalized increase in thyroid gland volume, termed goiter. In most patients, the entire thyroid gland can be increased up to 2 - 3 times above normal. Other hallmark features of the disease include thyroid eye disease termed Graves' ophthalmopathy (GO), and thyroid dermopathy termed pretibial myxedema. GO is the more common extra-thyroidal manifestation of GD and is clinically evident in 25 - 50 percent of the patients. The onset of GO may precede, coincide with, or follow the thyrotoxicosis. It is characterized by proptosis, periorbital and conjunctival edema, extraocular muscle dysfunction, and rarely, corneal ulceration or optic neuropathy. It can be a disfiguring and potentially sight-threatening autoimmune disorder. Thyroid dermopathy, as seen in pretibial myxedema, is a painless thickening of the skin, particularly over the lower tibia. It is due to the accumulation of glycosaminoglycans (GAG) and is a relatively rare occurrence in GD (2 - 3%).

GD is an autoimmune disease characterized by the presence of autoantibodies directed against the thyrotropin receptor (TSHR). These anti-TSHR autoantibodies (TRAB) mimic the action of TSH and activate the TSHR independent of its natural ligand. Receptor activation increases the downstream signal transduction with an increase in cyclic AMP (cAMP) production. There is growth and proliferation of thyrocytes and thyroid hormone T_3 and T_4 overproduction, leading to diffuse goiter and thyrotoxicosis. These TRAB with stimulatory activity are known as thyroid stimulating antibodies (TSAB) and is of IgG subtype.

1.1. Diagnosis of Graves' Disease

Clinical diagnosis is made based on the triad of goiter, GO and pretibial myxedema if present and confirmed through biochemistry by a combination of suppressed TSH and elevated free T_4. In early and recurrent Graves' disease, T_3 may be secreted in excess before T_4 is elevated. Therefore, if TSH is suppressed and free T_4 is not raised, serum T_3 should be measured. GD patients have autoantibodies against several thyroid antigens including thyroglobulin (Tg), thyroid peroxidase (TPO) and TSHR [8, 9]. Among these, TRAB is the pathogenic autoantibody and most critical in disease development. Testing of this autoantibody is useful in the diagnoses of 'apathetic' hyperthyroid patient or patient who presents with unilateral exophthalmos without obvious clinical features or laboratory manifestations of GD.

1.2. The Antigen in Graves' Disease: Thyrotropin Receptor (TSHR)

The TSHR is the primary antigen in GD. It is the target of both antigen-specific T cells and B-cell derived antibodies. The binding of its cognate ligand TSH or/and pathogenic TRAB changes the receptor and brings about the signal

transduction across the thyroid cell membrane. The TSHR has long been known to signal via cAMP signal transduction pathway. The receptor's cAMP signal transduction is regulated by TSH in a normal person. Growth and function of the thyroid are stimulated by cAMP which indirectly regulates the expression of the Tg and TPO genes. In Graves' disease, TSAB mimicking the action of TSH presents a continued stimulation of the cAMP pathway, thus causing hyperthyroidism (Figure 2). Conversely, inhibition of this cascade by autoantibodies such as thyroid-stimulation blocking antibodies (TSBAB) and thyrotropin binding inhibitor immunoglobulin (TBII) that block the TSHR would result in hypothyroidism.

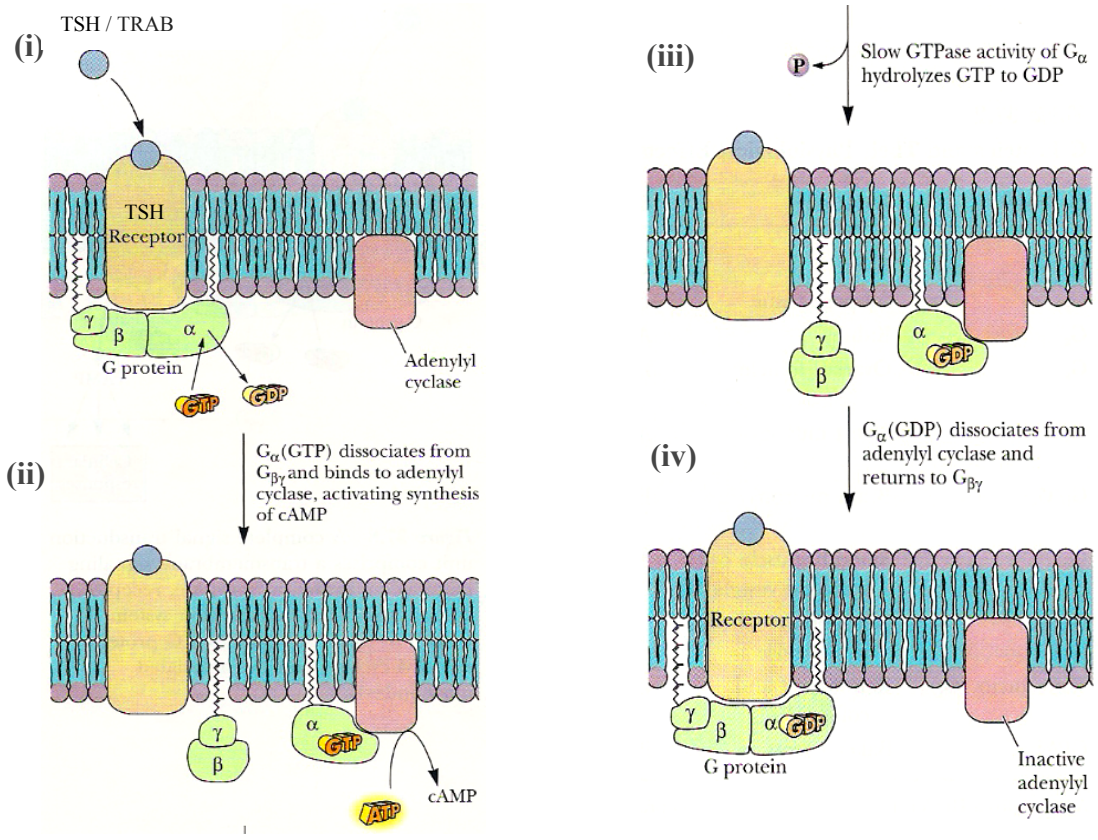


Figure 2. Activation of adenylyl cyclase following binding of TSH to TSHR.

(i) Following ligand binding to the receptor, a conformational change is induced in the receptor to catalyze a replacement of GDP by GTP on G_α.
(ii) The G_α-GTP complex dissociates from $G_{\gamma\beta}$ and binds to adenylyl cyclase, stimulating cAMP synthesis.
(iii) Bound GTP is slowly hydrolyzed to GDP by GTPase activity of G_α.
(iv) G_α-GDP dissociates from adenylyl cyclase and reassociates with $G_{\gamma\beta}$. G_α and G_γ are subunits linked to the membrane by covalent attachment to lipids [4].

The TSHR is the largest of all G protein-coupled receptors (GPCR) which consists of a large extracellular ligand binding domain linked to seven transmembrane segments, and an intracellular tail. It is found to be much more susceptible to constitutive activation by mutations, deletions, or even mild trypsin digestion than other GPCRs (Figure 3) [7].

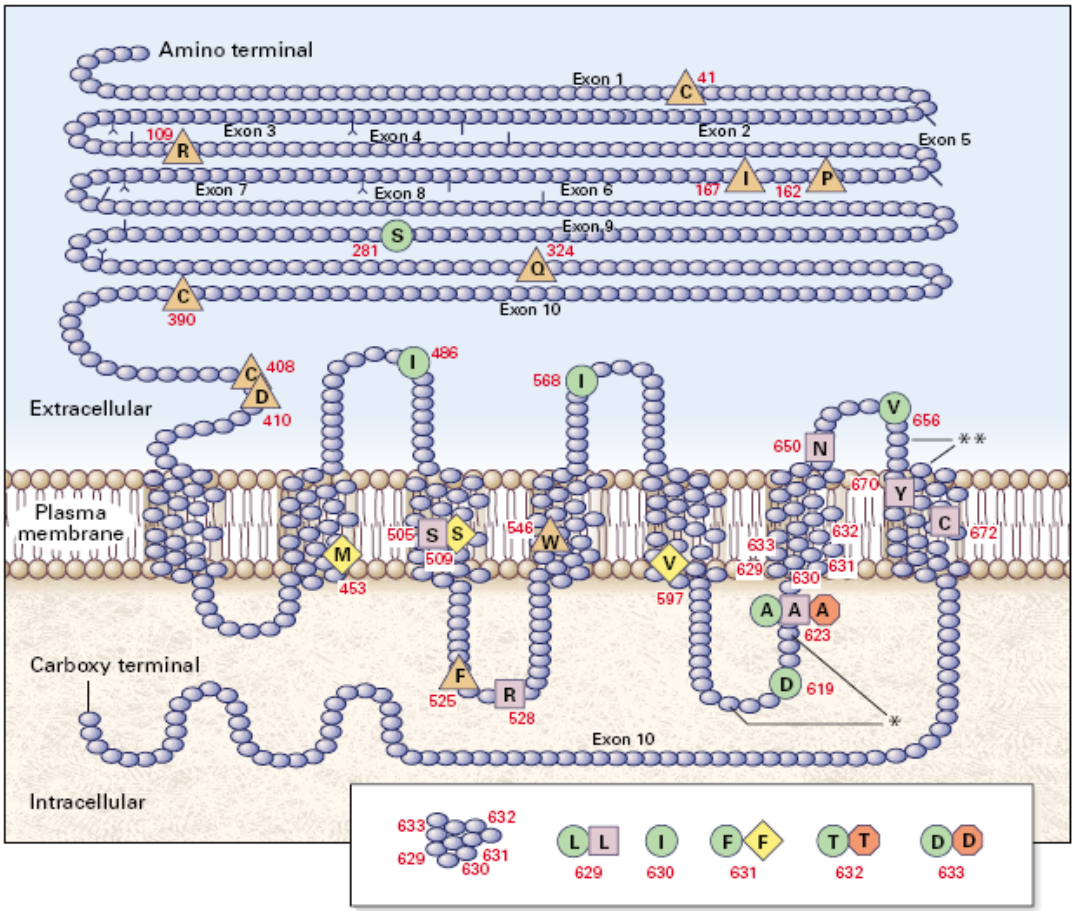


Figure 3. The Thyrotropin Receptor with known mutations marked.

Gain-of-function mutations are denoted by circles (●) in the case of hyperfunctioning thyroid adenomas, squares (■) in the case of familial autosomal dominant hyperthyroidism, diamonds (◆) in the case of sporadic congenital hyperthyroidism, and octagons (⬢) in the case of thyroid carcinomas. Loss-of-function mutations are denoted by triangles (▲). Letters indicate the amino acid in the wild-type receptor. The asterisk (*) and double asterisk (**) indicate deletions resulting in a gain of function in hyperfunctioning thyroid adenomas [7].

The TSHR is unusual among the GPCRs in that the single-chain TSHR undergoes intramolecular cleavage to form ligand-binding, disulfide-linked subunits A (α, N-terminal extracellular portion) and B (β, membrane bound). A segment of ~50 residues (C-peptide region) is removed from the N-terminal end of the B subunit (Figure 4(i)). This process also leads to the shedding of heavily glycosylated autoantibody-binding A-subunits from the cell surface which is preferentially recognized by TSAB (Figure 4(ii)). The shed A-subunits have been shown to bind TSH even without the B-subunit. These post-translational processes (cleavage and A-subunit shedding) are regulated by TSH [6]. Majority of the epitopes for TSAB are present on the N-terminal region between amino acid residues 25 and 165 of the extracellular domain while those for TSBAB and TBII are on the C-terminal region (between amino acid residues 261 and 370) [10]. However, recent studies using monoclonal antibodies on TSHR epitopes indicate a much closer overlap of TSAB and TSBAB binding sites [11].

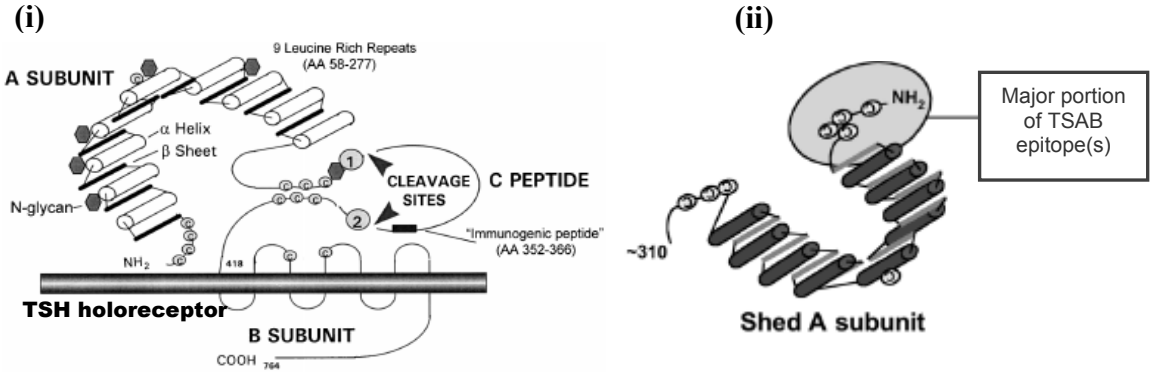


Figure 4. Schematic representation of different forms of the TSHR.

(i) Intramolecular cleavage of the single polypeptide chain is followed by removal of the C peptide region, with the A subunit remaining tethered to the membrane-spanning B-subunit by disulfide bonds.

(ii) The autoantibody-binding A-subunit [6].

1.3. TSHR Autoantibodies in Graves' Disease

TSHR autoantibodies (TRAB) show functional heterogeneity. Autoantibodies which mimic TSH action to stimulate thyroid hormone production are called thyroid-stimulating antibodies (TSAB), while those which block TSH actions are called thyroid-stimulation blocking antibodies (TSBAB). Antibodies that inhibit TSH binding to the receptor are called TSH-binding inhibitor immunoglobulin (TBII) [12]. GD patients have all three antibodies frequently coexisting in their blood (Figure 5). In general, TSAB should dominate over other TRAB during hyperthyroid phase of GD. They can also cause transient neonatal hyperthyroidism by transplacental crossing of IgG from mother to fetus. TSAB are restricted to the IgG subclass, while TSBAB are not restricted to a given subclass of immunoglobulin [13].

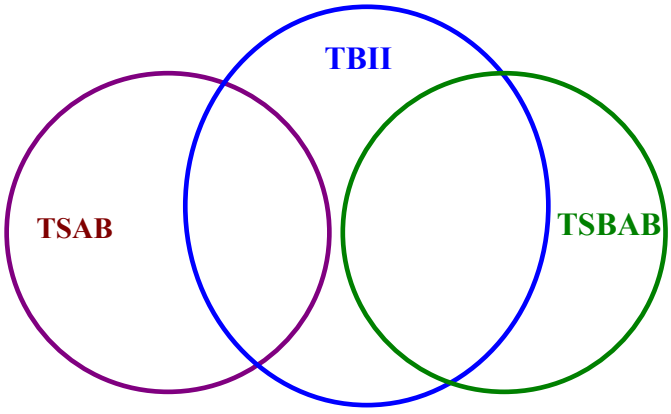


Figure 5. A schematic representation of the relationship between quantities of heterogeneous TRAB in Graves' disease [5].

1.4. Detection of TRAB

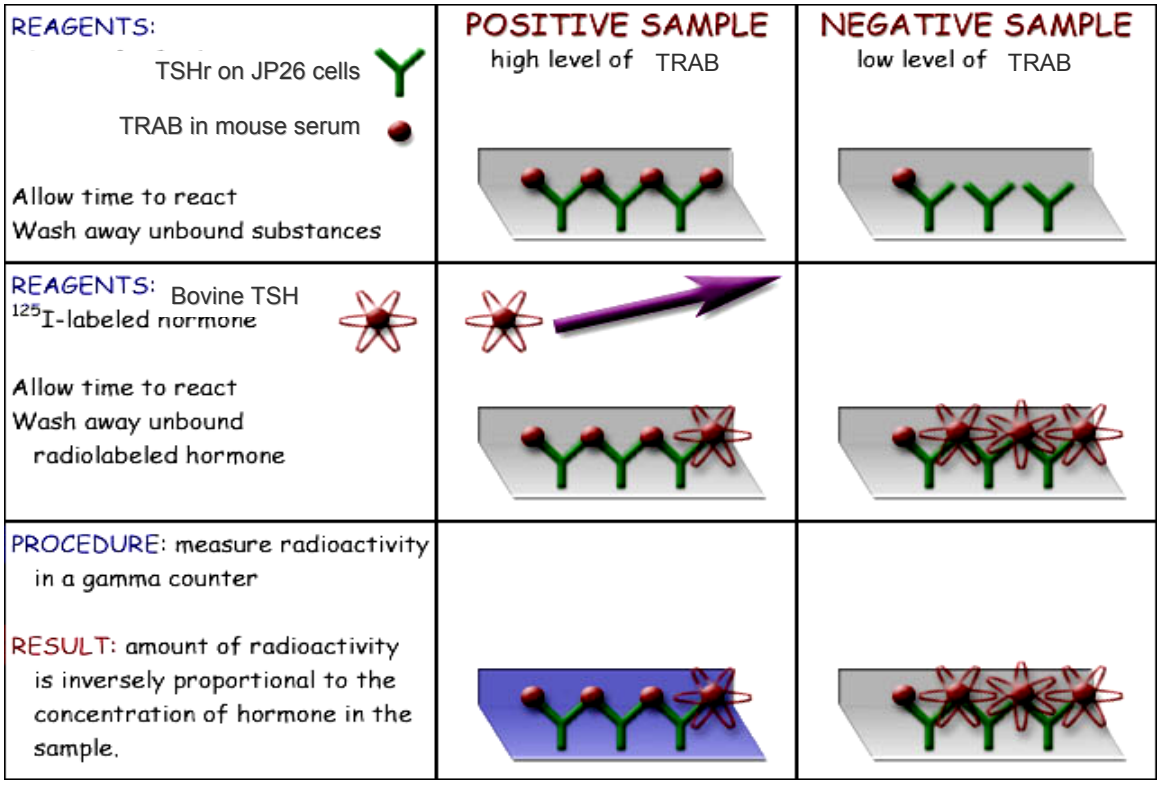
TRAB is useful for differential diagnosis of GD from other causes of hyperthyroidism, for follow up of patients with GD under treatment with antithyroid drugs, for the diagnosis of GO and for monitoring GD in pregnancy or after delivery. It can be detected and measured by 3 methods:

1. Indirect competitive assay (TBII assay),
2. Measurement of cAMP levels stimulation in the case of TSAB, or measurement of suppression of TSH-mediated cAMP production in the case of TSBAB,
3. Flow cytometry.

1.4.1. Indirect Competitive Assay (TBII Assay)

This is a competitive assay where TRAB and I^{125} labeled bovine TSH compete for the binding sites on the TSHR (Table 1). TRAB inhibit labeled TSH binding to the TSHRs in a dose- and time-dependent manner. This assay does not distinguish between stimulating and blocking TRAB.

Table 1. TSH Binding Inhibitory Immunoglobulin (TBII) Assay



1.4.2. TSAB and TSBAB Assays

Interaction of stimulating TRAB at the TSHR results in cAMP production as shown in Figure 2. TSAB assay is carried out by measuring the amount of cAMP generated from incubation of stimulating TRAB with cells expressing TSHRs over a measured period of time. TSBAB is similarly performed except that in this case, incubation of blocking TRAB is done in the presence of TSH and cells expressing TSHRs. Since blocking TRAB inhibits TSH, a reduction of TSH-mediated cAMP generation is detected. Cyclic AMP can be measured in the intra- or extra-cellular compartment and is usually done with a radioimmunoassay kit.

1.4.3. Detection of TRAB by Flow Cytometry

In this method, cells expressing TSHRs are incubated in the presence of TRAB-positive sera and detection is done by a secondary antibody conjugated with a fluorescein dye. Cells prepared in this manner are then put through a fluorescence-activated cell sorter (FACS) (Figure 6). The cell stream that is passing out of the chamber is encased in a sheath of buffer fluid and illuminated by a laser. Each cell is measured for size (forward light scatter) and granularity (90^{o} light scatter), as well as for presence of colored fluorescence. Thus by measuring the fluorescence intensity of each cell after interrogation by a laser beam, the machine is able to distinguish TRAB bound and non-TRAB bound cells (Figure 7).

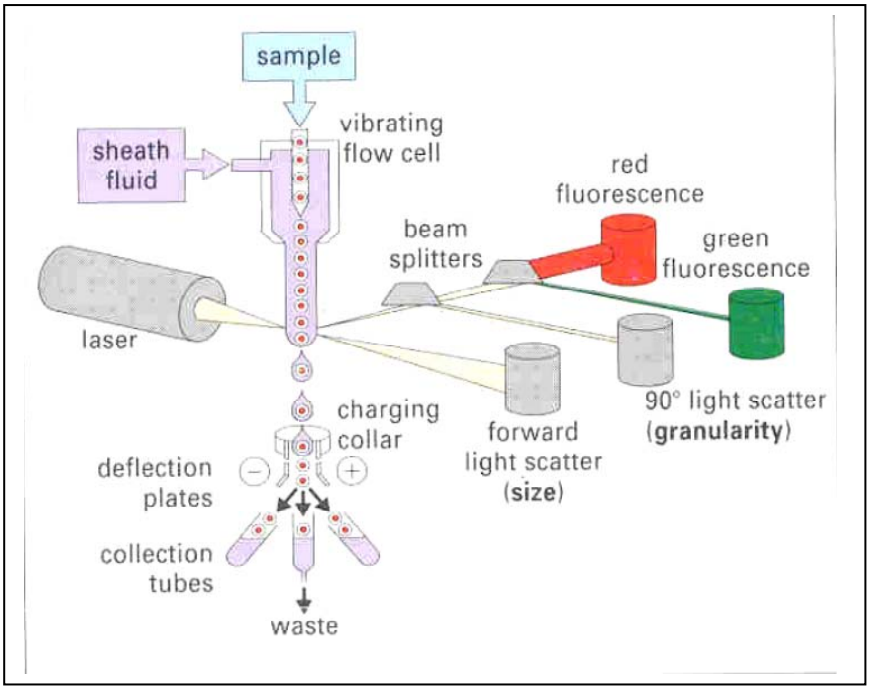


Figure 6. FACS, Fluorescence Activated Cell Sorter

TRAB positive and TRAB negative sera can be identified based on their fluorescent brightness [2].

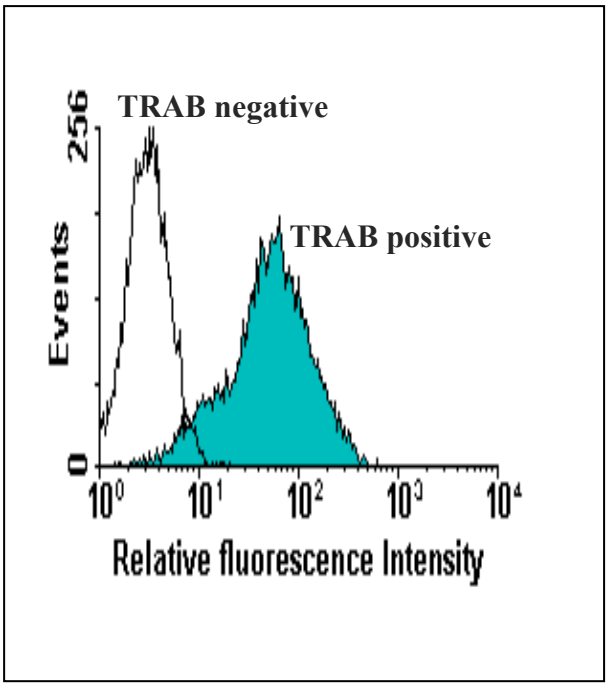


Figure 7. Histogram for TRAB bound and unbound population

Y-axis denotes number of cells while X-axis showed fluorescence for two populations.

2. Graves' Ophthalmopathy (GO)

Graves' Ophthalmopathy is a potentially disfiguring and sight-threatening component of GD. Although clinically evident only in 25-50%, almost all patients with GD have some degree of ocular changes that can be detected by more sensitive methods such as ultrasonography, computed tomographic, or magnetic resonance imaging. Clinical features of GO result from changes in the orbit that consists of i) orbital inflammation, ii) swelling in the retrobulbar space, and iii) restriction of extraocular muscle motion and/or impairment of optic nerve function.

Swelling in the retrobulbar space is due to accumulation of glycosaminoglycans (GAG) by the orbital fibroblasts. GAG is intensely hydrophilic and binds water causing gross enlargement of the extraocular muscles and edema of the surrounding connective tissues. This increase in tissue volume within the confines of the bony orbit gives rise to proptosis, a forward displacement of the globe [14]. Restriction of extraocular muscle motion initially occurs as a result of swelling. At a later stage, fibrosis and atrophy due to chronic compression and inflammation set in [15]. In addition to the accumulation of GAG, mononuclear cells infiltrate the orbital tissues [16]. On histologic examinations, besides the expansion of eye muscle and orbital fat tissues, lymphocytic infiltrate consisting of predominantly $CD4^+$ and $CD8^+$ T cells with a few B cells can be seen. Once stimulated, the T cells release numerous cytokines which bring about orbital fibroblast proliferation, induction of glycosaminoglycan synthesis and transformation of orbital preadipocyte fibroblasts into orbital fat cells. Therefore, GO is fundamentally, an inflammatory disease of the orbital tissues [15, 17, 18].

2.1. T Lymphocytes (T cells) Development

T cells are lymphocytes that arise from stem cells in the bone marrow. They leave the bone marrow at an immature stage and complete their development in the thymus. Most T cells in the body belong to one of two subsets, $CD8^+$ or $CD4^+$ and their development in the thymus can be traced by surface markers. In the thymus, the cells initially possess both $CD8^+$ and $CD4^+$ markers, making them double positive cells. They eventually loose either the $CD4^+$ or $CD8^+$ marker to become one of the functional subsets.

All T cells possess antigen receptor molecules on their surfaces called T cell receptor (TcR). Antigens are the obligatory first signals for lymphocyte activation. Chemically different antigens stimulate different types of immune response. TcRs recognize antigens only when they have been ingested, degraded and presented on the surface of an antigen presenting cell (APC). Antigens are bound to specialized antigen-presenting glycoprotein called major histocompatibility complex (MHC) molecules on the surface of the APC. On contact with antigen presented by MHC on APC, T cells are activated [3].

$CD8^+$ cells are cytotoxic T cells (Tc cells) and they secrete molecules that destroy the cell to which they are bound (Figure 8). $CD8^+$ T cells are activated by antigen peptides presented by MHC class I molecules, and are directed to destroy the APC by inducing them to undergo apoptosis. Most cells express MHC class I molecules and therefore can present pathogen-derived peptides to $CD8^+$ T cells if infected with a virus or other pathogen that penetrates the cytosol. $CD8^+$ T cells are specialized to respond to intracellular pathogens [3].

$CD4^+$ T cells activate B cells towards antibody responses and macrophages towards microbial destruction. They also recruit these cells to the site of infection

through cell-cell interactions and cytokine production. They are essential for both the cell-mediated and antibody-mediated branches of the immune system. $CD4^+$ T cells recognize and are activated by antigens presented by MHC class II molecules on specialized APCs such as dendritic cells, macrophages and B cells, which take up and process material from the extracellular environment. Because their function is principally to help other cells achieve their effector functions, they are often called helper T cells (Th cells). $CD4^+$ T cells can be further subdivided into helper T cell 1 (Th1) and helper T cell 2 (Th2) [3].

2.1.1. Helper T cells

When helper T cells are activated by dendritic cells, they can differentiate into either Th1 or Th2 effector cells. Helper cells secreting cytokines that mainly activate macrophages and B cells with production of opsonizing antibodies of IgG1 subclass are called Th1 cells. Helper cells helping primarily in B cells antibody responses are called Th2 cells (Figure 8). While Th2 cells work within secondary lymphoid tissues, Tc cells and Th1 cells must travel to the site of infection to carry out their functions [3].

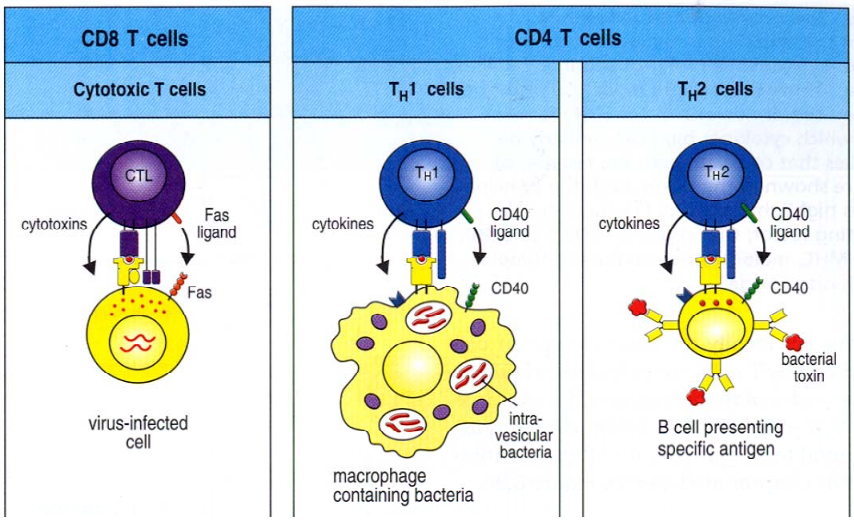


Figure 8. Three classes of effector T cell specialized to deal with three classes of pathogens.

$CD8^+$ cytotoxic T cells kill cells that present peptides derived from viruses and other cytosolic pathogens. Th1 cells recognize peptides derived from pathogens or their products that have been swallowed by macrophages. Th2 cells activate naïve B cells and control many aspects of the development of antibody response [3].

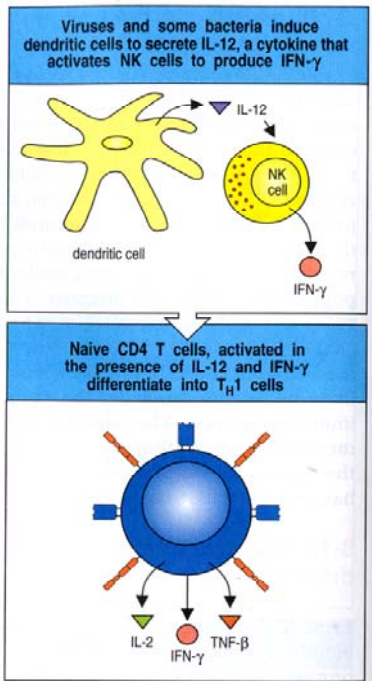


Figure 9. Activation of helper T cell and differentiation into Th 1 cells.

IFN γ produced by NK cell that was stimulated by IL-12 produced by dendritic cell, causes naïve $CD4^+$ T cells to differentiate into Th1 cells [3].

Cytokines produced during infection or inflammation modulates the differentiation of helper T cells into Th1 or Th2 responses. Interleukin 12 (IL-12) produced during the early stage of infection, is mainly the product of dendritic cells and macrophages. It stimulates natural killer (NK) cells to produce gamma interferon (γIFN), which in turn stimulate differentiation of naïve $CD4^+$ T cells into Th1 cells and activates macrophages (Figure 9). In addition, IL-12 and γIFN also inhibits the development of Th2 cells. Conversely, differentiation of naïve $CD4^+$ T cells towards Th2 response is promoted by IL-4 which is produced by subsets of T cells and mast cells. IL4 also has the property of inhibiting Th1 cell differentiation. The commitment of the $CD4^+$ T cell response towards a Th1 or a Th2 phenotype probably depends on the way the antigens interact with immature dendritic cells, macrophages, and NK cells during the early phases of an infection/inflammation and the profile of cytokines that is synthesized at that time. The cytokines produced by effector Th 1

and Th 2 cells also tend to suppress each other's differentiation, so that once a $CD4^+$ T cell response has been pointed in one direction, this bias becomes reinforced (Figure 10).

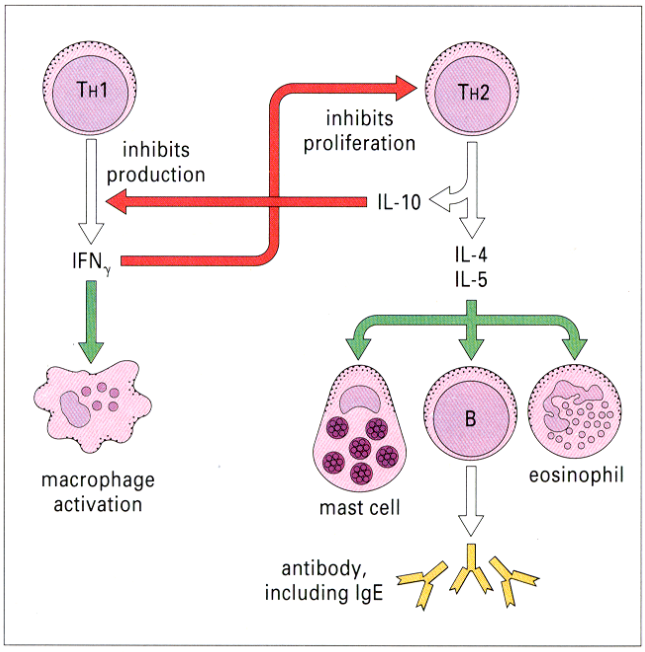


Figure 10. Suppression of Th cell by another Th cell which has been activated. Cytokines produced by one Th cell switches off the production of cytokines by the other Th cell, thus only one Th cell can be activated at a time [2].

2.1.2. Cytokines

Cytokines are soluble proteins secreted by T cells and other cell types in response to activating stimuli. Cytokines mediate many effector functions of the cells that produce them. They are the principal mechanisms by which various immune and inflammatory cell populations communicate with one another.

The cytokines secreted by Th 1 cells include γIFN, GM-CSF, TNF α, TNF β, IL2, IL3, CD40 ligand, and Fas ligand. They bias towards macrophage activation, which leads to inflammation and a cell-mediated immune response, dominated by cytotoxic $CD8^+$ T cells and/or $CD4^+$ Th 1 cells, and macrophages. Figure 11 shows a summary of the activities of cytokines produced in Th1 responses.

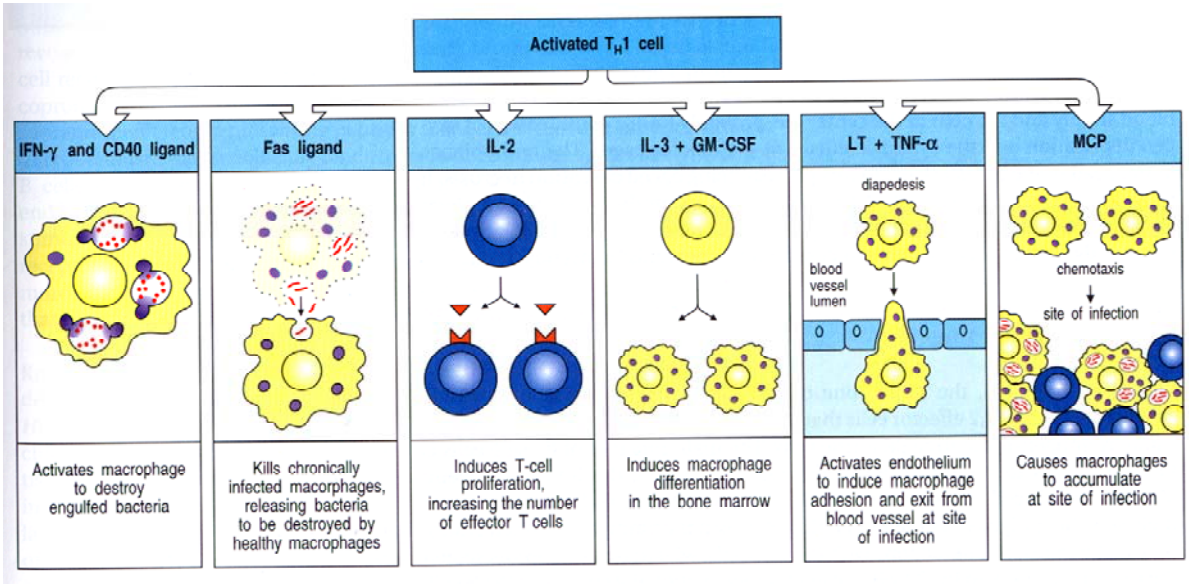


Figure 11. Activities of an activated Th1 cell

Activation of Th1 cells results in the synthesis of cytokines. The six panels show the effects of different cytokines. LT - lymphotoxin. MCP - macrophage chemoattractant protein [3].

Cytokines secreted by Th 2 cells, in contrast, induce mainly B-cell differentiation and antibody production. They include IL3, IL4, IL5, GM-CSF, IL10, TGF β, Eotaxin, and CD40 ligand and they mediate the processes of humoral immune response. This division of labor is not absolute, however, because Th 1 cells have some influence on antibody production.

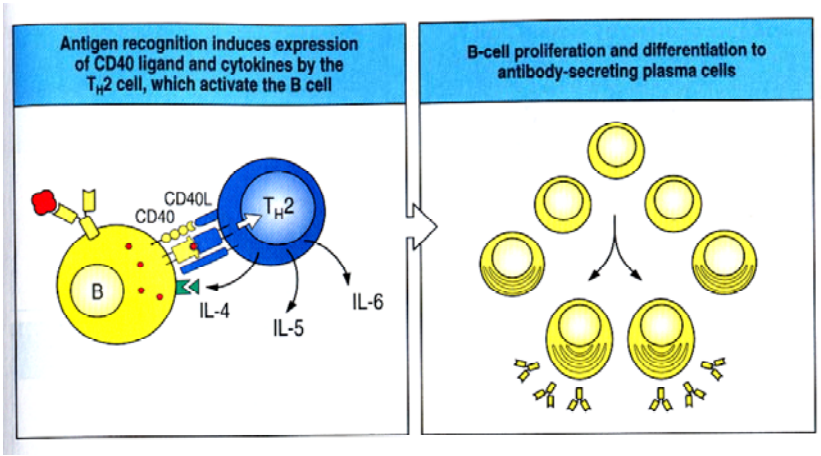


Figure 12. Th2 cells acting on naive B cells.

Stimulation of naïve B cells led to proliferation and differentiation to form plasma cells dedicated to the secretion of antibody [3].

2.2 Helper T cell Involved in Graves' Ophthalmopathy

The inflammatory responses occurring in the orbits of GO patients have been studied extensively. Characterization of T cell populations and cytokine profiles present in orbital tissues often yield contradicting results. A study by Pappa et al reported the predominance of $CD4^+$ T cell lines derived from extraocular muscles of GO patients and that both Th1 and Th2 cytokine profiles were present in their T cell lines [19]. Other reports showed predominance of $CD8^+$ T cells in the orbit with either inconsistent cytokine profiles, a mixture of Th1 and Th2 responses or predominance of Th1 profile [20, 21]. The fundamental aim underlying these studies is the question whether cell-mediated immunity (Th1) or humoral immunity (Th2) is the major effector of the inflammation present in GO [22-24]. These studies into the balance of Th1 and Th2 responses are often confounded by problems highlighted below which make accurate interpretation of results difficult.

- Difficult accessibility of orbital tissues – samples of orbital fat and muscles are obtained mostly at the time of surgical interventions, which are usually performed in the late stages of the disease when the active inflammatory reaction caused by the initial autoimmune attack has disappeared and fibrosis dominates the picture.
- Differing techniques of investigation – In some studies, culture of T cell lines and T cell clones in the presence of IL2 or IL 2 and IL4 could potentially bias towards detection of either Th1 or Th2 responses respectively. In this case, the populations of T cultured cells may not be truly reflective of the *in-situ* composition [23, 25-27].
- Differing genetic background – GO tissue samples derived from patients are heterogeneous in their genetic makeup. A complex network of genetic factors

governs the response of the immune system. Genetic factors such as HLA, T cell regulatory gene, polymorphisms in cytokine, cytokine receptors, and toll-like receptors have been shown to determine the type and magnitude of immune responses and may be important in the pathogenesis of both infective and autoimmune diseases [28-31].

2.3. Animal Model of Graves' Ophthalmopathy

The development of an animal model of GO will to an extent avoid the limitations encountered in previous studies, although it is recognized that disease pathogenesis in animal models may differ significantly from that in human disease and therefore may not be directly applicable. Experimental animals can be chosen for their genetic composition. Tissue sampling can be done at specific time of onset of the disease and these samples will be naïve to all forms of therapeutic intervention. In recent years, significant progress has been made in establishing a mouse model of GO. Orbital inflammation has been observed in 2 models: 1) after genetic immunization of NMRI outbred mice treated with full length TSH receptor in an eukaryotic expression plasmid [32] and 2) after transfer of TSH receptor sensitized T cells in Balb/c mice [33]. In this current project, I used the method of genetic immunization for achieving the objective of inducing inflammatory responses in the orbit of immunized animals.

The GD mouse model with orbital inflammation was first successfully generated through genetic immunization with full length TSHR by Costagliola et. al. [32]. The outcome was a strong humoral response where all the immunized outbred mice produced antibodies capable of recognizing the recombinant receptor expressed at the surface of stably transfected Chinese hamster ovary (CHO) cells (JP19 cells) in

flow cytometry, and most had detectable levels of TSBAb activity in their serum. Five of 29 mice that were injected showed sign of hyperthyroidism with elevated total T4 and suppressed TSH levels. In these 5 hyperthyroid mice, thyroid-stimulating activity was detected in the serum and there was development of goiter with extensive lymphocytic infiltration, (Figure 13)

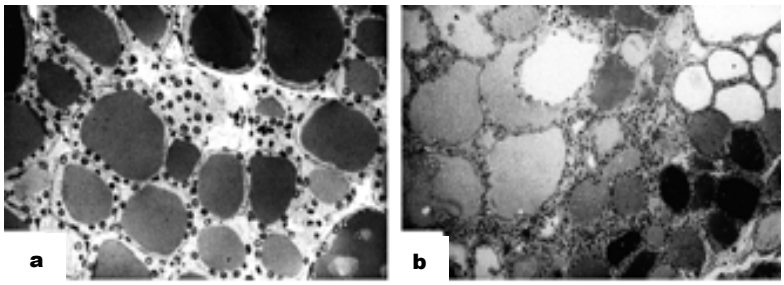


Figure 13: Semi-thin section of the thyroid from (a) control NMRI mouse. x160 and (b) of thyroids immunized hyperthyroid NMRI mice, showing very extended inflammatory infiltrate among the heterogenous follicles. x320 (31).

and these animals displayed ocular signs suggestive of GO (Figure 14) including edema, deposit of amorphous material and cellular infiltration of their extraocular muscles.

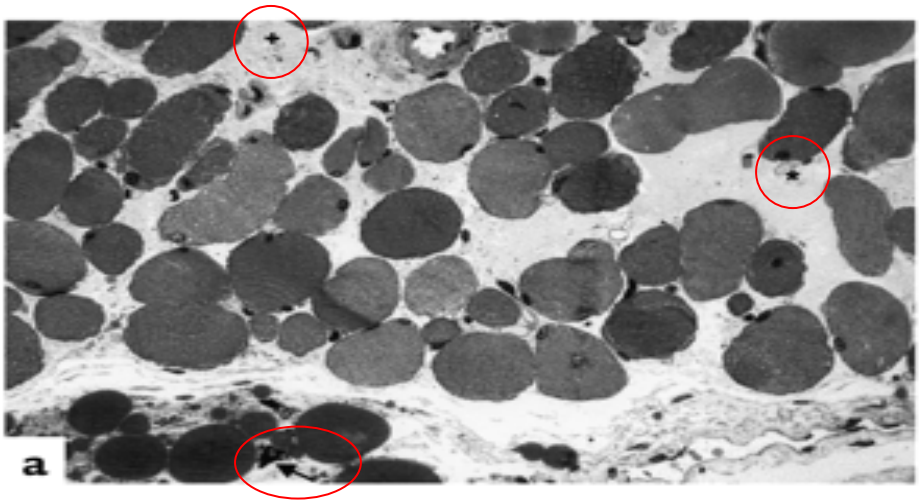


Figure 14: **Semi-thin sections of extraocular muscles from immunized hyperthyroid NMRI mice.** (a) The muscular cells, in transverse section, are dissociated by an edema and a deposit of an amorphous material (*) or by fibrous tissue (+). x250. The adipose tissue infiltrating the muscle is made of cells of various sizes, often in association with mast cells (arrows).

These signs, reminiscent of features of GD and GO, demonstrated that genetic immunization of outbred NMRI mice with human TSHR provided the most convincing and closest animal model available at that point in time for GD.

The other mouse model of GD with orbital inflammation, generated by Many et. al [33], was induced by transfer of TSHR sensitized T cells in Balb/c mice into syngeneic mouse. Of the 35 Balb/c mice experimented, thyroiditis was induced in 60-100% and the lymphocytic infiltrate comprised of activated T and B cells. Immunoreactivity for IL-4 and IL-10 was present. Autoantibodies to the receptor such as TBII, were also induced. A total of 17 of 25 Balb/c mouse orbits examined displayed changes which consisted of accumulation of adipose tissue, edema caused by periodic acid Schiff-positive material, dissociation of the muscle fibers, presence of TSHR immunoreactivity, and infiltration by lymphocytes and mast cells. (Figure 15)

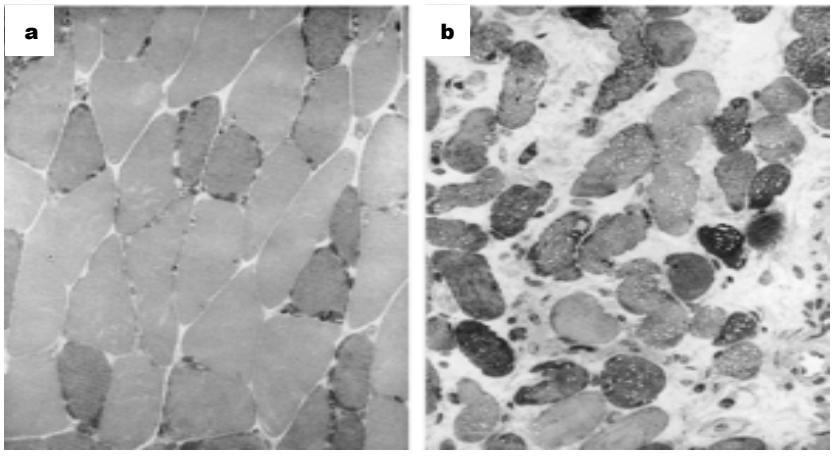


Figure 15: Balb/c ocular muscle. (a) Balb/c recipients of nonprimed T cells. The histology is normal with intact muscle fibers. x320. (b) Balb/c recipient of TSHR-primed T cells 12 wk after transfer. Organization of muscle bundles has been lost with individual muscles being dissociated by edema. x320.

2.3.1. Balb/c inbred versus Swiss Outbred mice

Balb/c mice are inbred strain which is produced by NUS animal holding unit. The strain is obtained through 20 or more consecutive generations of brother and sister matings with all individuals being traced from a common ancestor in the 20th or subsequent generation. Inbred strains are more uniform, better defined and genetically more stable than outbred mice. This strain remains genetically stable for many generations. In contrast, for Swiss Outbred mice, brother and sister mating is avoided with the aim to maintain as heterogeneous as possible the animal population. In this strain, the inbreeding coefficient adopted is less than 1%. Swiss outbred mice is a general-purpose mouse recommended for dissection and any work not requiring the special qualities of inbred strains.

Differences between inbred and outbred mouse responses to immunization had been reported previously. Where genetic immunization using TSHR cDNA [34] and transfer of TSHR sensitized T-cells [33] were used in inbred strain, thyroiditis was induced in 60-100% of the mice. Autoantibodies recognizing the native receptor were detected in virtually all mice sera but most displayed blocking TSBAb and TBII activities. No hyperthyroidism was observed. When genetic immunization using TSHR cDNA was used to generate the mouse model in outbred strain [32], hyperthyroidism, with elevation of total T4 and suppression of TSH levels, was demonstrated in 1 out of 5 mice. These mice had stimulating TSAb activity,increased thyroid mass with extensive lymphocytic infiltration and histological evidence of thyroid follicular cell hyperplasia.

2.3.2. Genetic Immunization

Genetic immunization, also known as DNA vaccination, represents a novel approach for achieving specific immune activation. It has been known for decades that delivery of naked DNA into an animal could lead to *in vivo* gene expression. The concept behind genetic immunization is simple. The gene encoding an antigen is cloned into a plasmid with an appropriate promotor, and the plasmid DNA is administered to the vaccine recipient by injection into the subcutaneous tissue or muscles. The injected DNA is transfected into the dendritic cells or keratinocytes of the host and the latter are thought to be reservoirs for the antigen. The resultant foreign protein is produced within the host cell and then processed and presented appropriately to the immune system, inducing a specific immune response. Immunization with DNA thus mimics live infection, with the antigen synthesized endogenously by host cells. This synthesis leads to the induction of a cytotoxic T cell response via the MHC class I-restricted pathway. Concurrently, antigen is released extracellularly and this process primes the induction of a humoral response, by way of Th response via MHC class II-restricted antigen presentation by APCs that have taken up the foreign antigen (Figure 16).

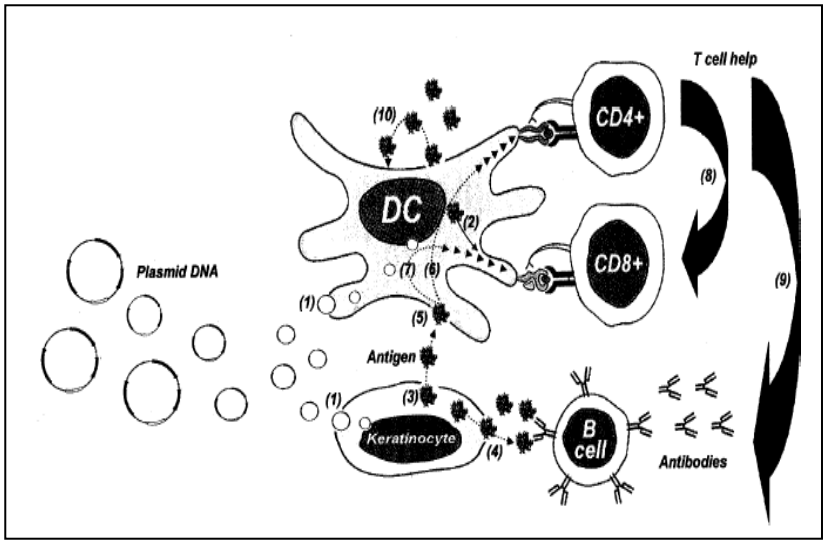


Figure 16. Plasmid DNA immunization.

Plasmid injected intra muscularly, transfect dendritic cell and keratinocyte. Antigen presented to naïve $CD4^+$ T cell. T cell activated and differentiates to effector T cells which activate $CD8^+$ and B cells.

2.3.3. Timing of blood and tissue sampling

In a study by Tang et al [35], genetic immunization of gene encoding protein of interest was used as a method to elicit an immune response in mice. Young mice (8-15 weeks old) were used and antibodies directed against gene of interest were detectable in most mice within 2 weeks of first immunization. The study concluded that primary response could be augmented by a subsequent 2nd and 3rd DNA boosts although there was no recommendation on the timing interval of these boosters. In our study, the 2nd and 3rd DNA boosts took place at day 28 and day 56 respectively after the initial immunization and blood sampling was done 5 days prior to initial immunization and at sacrifice at day 112 for detection of sera antibodies against the TSHR. These time lines followed the immunization protocol described by Costagliola et al [32]. In a previous publication by Costagliola [36], histological changes showing atypical lymphoblastoid infiltration and follicular destruction of thyroids was already observed at day 49 after immunization with extracellular domain of the human TSHR in Balb/c mice. In another publication also by Costagliola [32], using genetic immunization of outbred NMRI mice with cDNA encoding the human TSHR, changes in the thyroid with extensive lymphocyte infiltration and ocular signs suggestive of GO were also seen in sectioned and stained tissues at day 112 during sacrifice.

2.4. Cytokine Profile Study using Real-Time PCR, TaqMan® Technology

Real-Time PCR is a sensitive and specific means of quantifying a gene of interest. It has the ability to monitor the progress of the PCR as it occurs because data is collected throughout the PCR process, rather than at the end of the PCR. In real-time PCR, reactions are characterized by the point in time during cycling when

amplification of a target is first detected rather than the amount of targets accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed (Figure 17).

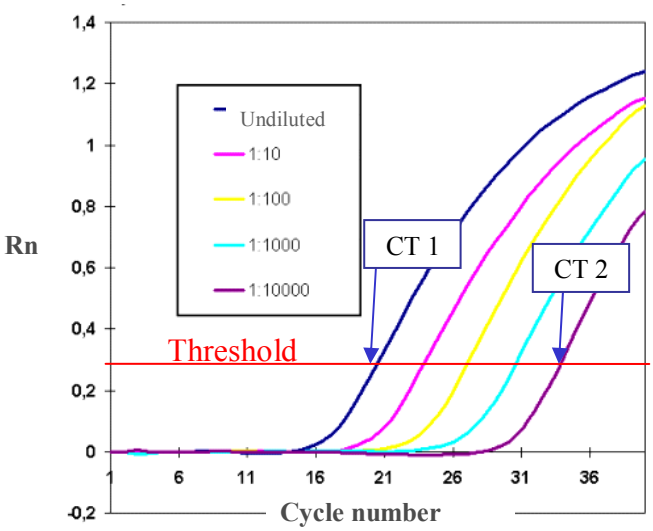


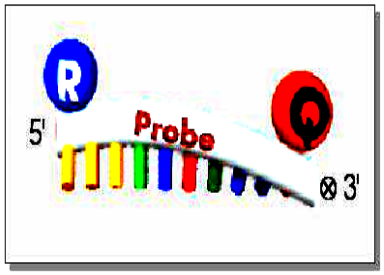
Figure 17. Amplification curve

Rn is the measure of reporter signal. Threshold is the point of detection. Cycle threshold (CT) is the cycle at which sample crosses threshold. CT1 which is more concentrated requires fewer cycles for fluorescence detection as compared to CT2.

The fluorescence-monitoring system for DNA amplification used in our experiments is TaqMan® probe. The probe consists of a short single strand of polynucleotide linking 2 fluorophores (Figure 18). When in close proximity, before the DNA polymerase acts, the quencher (Q) fluorophore reduces the fluorescence from the reporter (R) fluorophore by means of fluorescence resonance energy transfer (FRET). This is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5' end of the probe and the quencher at the 3' end.

Figure 18. TaqMan® probe

The red circle represents the Quencher that suppresses the emission of signal from the Reporter dye (blue circle) when in close proximity. Picture taken from www.appliedbiosystems.com



Once the TaqMan® probe has bound and the primers anneal to specific places at the DNA template, *Taq* polymerase then adds nucleotides and displaces the Taqman® probe from the template DNA. This allowed the reporter to break away from the quencher and to emit its energy, which is then quantified by the instrument (Figure 19). The greater the number of cycles of PCR takes place, the higher the incidence of Taqman® probe binding and this in turn causes greater intensity of light emission.

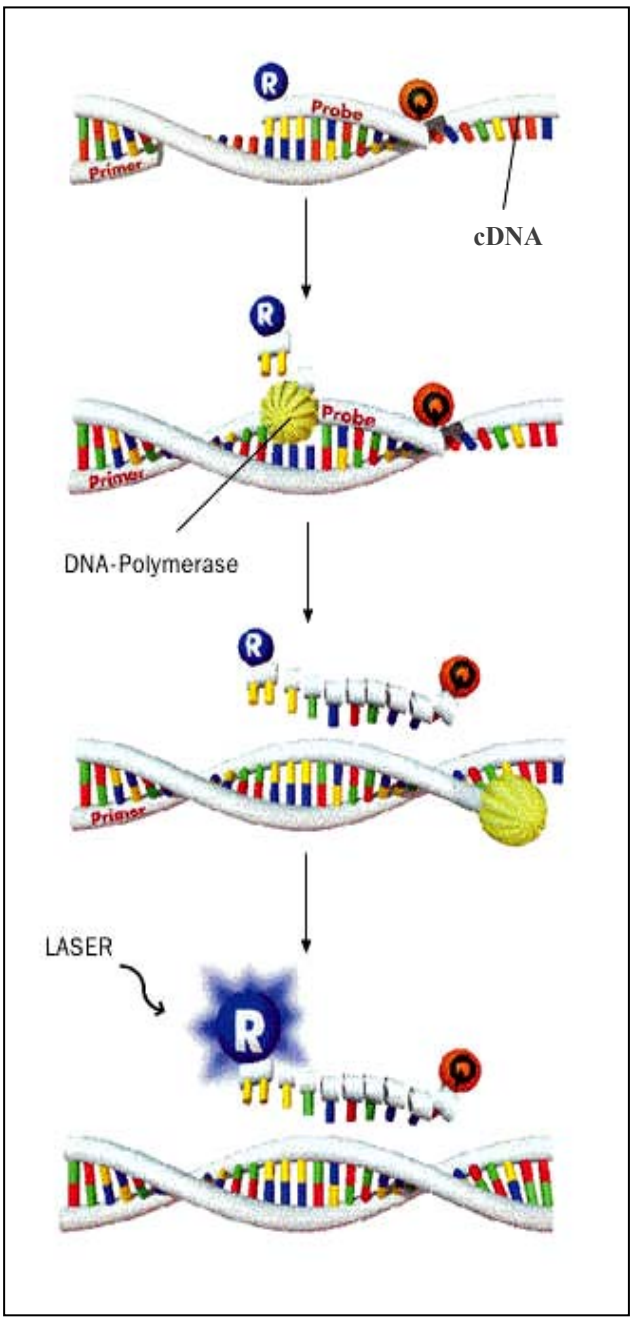


Figure 19. Principles of TaqMan®

First there is specific annealing of probe and PCR primers to the cDNA. Then, primer extension by *Taq* DNA polymerase causes hydrolysis of TaqMan® probe. Probe is cleaved and displaced from template. Once the probe is cleaved, the reporter dye is allowed to emit its energy which can be detected by the machine. The signal increases in proportion to the number of cycles performed. Picture taken from www.appliedbiosystems.com

Relative quantitation is a method of quantitation where the amount of target gene expression in a sample is expressed in relative quantity to another sample. The latter, known as a calibrator, can either be an external standard (serial dilution of a positive sample) or a reference sample (a negative sample or untreated sample). Results of such quantitation are expressed in target to reference ratios. A control gene, usually a housekeeping gene (e.g. β-actin, ribosomal RNA, GADPH) is co-amplified in the same tube in a multiplex assay in order to correct for sample-to sample variation in input material. Such control genes that may also serve as positive controls for the reaction. An ideal control gene is one which is expressed in a uniform fashion regardless of experimental conditions, sample treatment, origin of tissue/cell types, and developmental staging. The comparative Ct (cycle threshold) method is used to calculate changes in gene expression as relative fold difference between an experimental sample and a calibrator sample using the formula $2^{-\Delta\Delta Ct}$ where 2 is the 'efficiency' of the amplification, $\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator), and ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene.

For example, one may wish to evaluate the change in a particular gene expression (S) in treated and untreated samples. For this hypothetical study, one can choose the untreated sample as the calibrator sample and a housekeeping gene (H) to normalize input amount of RNA material. For both treated and untreated samples, the Ct values of both target and housekeeping genes can be obtained and ΔCt calculated by the formula: $(Ct_S - Ct_H)$ which is the difference between target gene and housekeeping gene. The $\Delta\Delta Ct$ is then subsequently obtained by subtracting ΔCt of treated sample from that of calibrator sample, i.e. the untreated sample. The relative fold change between the two samples is then obtained using the formula of $2^{-\Delta\Delta Ct}$.

II. AIMS OF STUDY

GO is an important and frequent complication of GD. It not only affects quality of life of patients but can be potentially sight threatening. There is currently no option of preventive treatment and management consists mainly of amelioration of inflammatory processes which are usually well underway once clinical presentations become overt. The pathogenesis of GO remains controversial and the study of the inflammatory processes and cytokine profiling in human tissue samples fraught with difficulties as highlighted in the earlier sections. Knowledge of the events in the immunopathogenesis of GO is required if the use of specific immunological interventions is desired. Therefore, understanding the nature of cytokine events is important in ameliorating or even halting the onset of GO. Cytokines effects can be blocked not only by corticosteroids but also by antagonists such as IL-1 receptor antagonists. Indeed, specific immunomodulatory therapies (anti TNF-α and other anti-cytokines) have shown promise in treatment of other immune diseases such as Crohn's disease and rheumatoid arthritis [37, 38]. These treatment modalities may prove to be valuable to GO as well since there is currently no satisfactory and effective therapy available to stop the progression of this disabling and sight-threatening illness. Therefore, a mouse model of GD and GO presents a unique and attractive opportunity to study the immunological events following immunization of animals with human TSHR in a controlled and specific manner. In this study, the following objectives were undertaken:

1. To genetically immunize 2 strains of mice, specifically Swiss outbred and Balb/c inbred mice, with the human TSHR.

2. To evaluate the production of anti-TSHR antibodies (TRAB) by measuring the TBII, TSAB and TSBAB in these immunized animals
3. To obtain relative quantities, by real-time PCR, of Th1 and Th2 cytokines present in the thyroidal, splenic and orbital tissues of mice immunized with the human TSHR and controls which were injected with empty plasmid.
4. To correlate the changes in cytokine gene expression with various TRAB measurements.

III. MATERIALS AND METHOD

1. Animal Experimentation

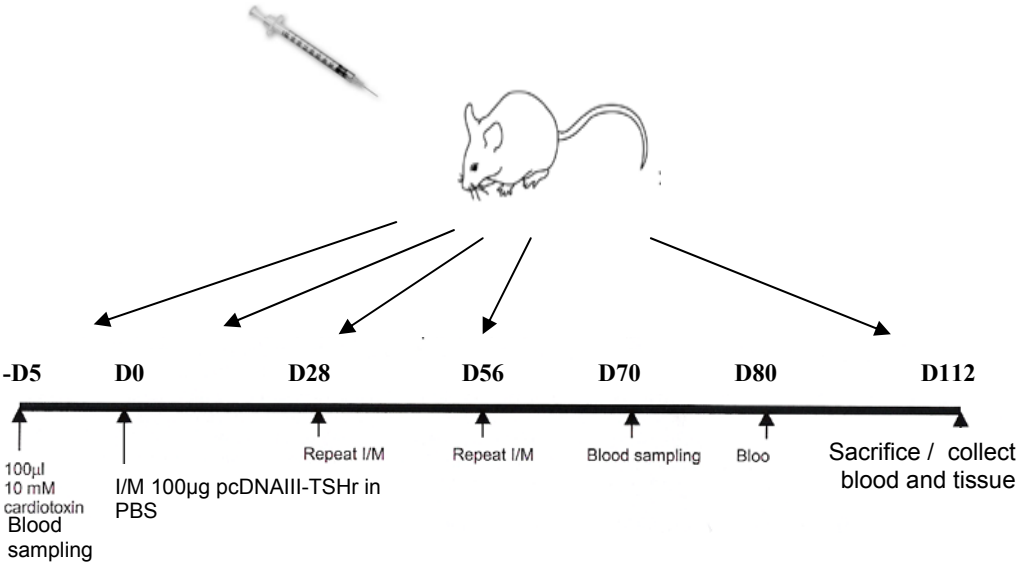


Figure 20. Schedule for genetic immunization and blood/tissue collection

Two strains of 6-7 weeks old female mice, Swiss outbred and Balb/c inbred, were studied. We immunized 20 Balb/c and 15 Swiss outbred mice. Five from each strain served as controls, while 15 and 10 mice respectively in each strain were immunized with TSHR. From previously reported studies [32, 34] almost all mice immunized generated antibodies against the native TSHR. Seventy five percent of these mice were positive for blocking TSBAb antibody while 17% positive for stimulating TSAb antibody. This gives the probability of obtaining 19 and 14 mice positive for TSHR antibody in inbred and outbred strains respectively for the study. Moreover, sample size of 15 to 20 mice was an optimal number for efficient experimental and tissue handling i.e, immunization, blood sampling and tissue RNA preservation during sacrifice. Given the reported 17% rate of hyperthyroidism [32]

on biochemical testing, the study cohort would generate 2 to 3 hyperthyroid mice. However, we believe that RT-PCR is a far more sensitive method for detection of cytokine changes and will be able to detail alterations in cytokine profile between control and test animals.

Experimental mice were injected on Day 0 in the anterior tibialis muscle with 100 μg of human TSHR cDNA in pcDNA3 plasmid dissolved with PBS after pretreatment 5 days earlier (- Day 5) with 100μl cardiotoxin 10μM, purified from venom of *Naja nigricollis;* (Latoxan, Valance, France). Control mice were injected with empty plasmid. Injections were repeated on D28 and D56 after the first immunization [34]. Blood samples of ~ 200μl were collected via tail vein before the first injection (-D5) and subsequently at sacrifice by cardiac puncture on D112. Sera samples were used for testing of TRAB antibodies and thyroid hormone levels. During sacrifice, the thyroid lobe, the contents of the orbit and the spleen were removed and preserved with RNA*Later*™ for RNA extraction. This was used subsequently for cytokine profile study using Real-Time PCR technique.

2. Sera Characterization

Blood collected on – Day 5 and Day 112 were spun down and the sera used for TRAB detection using methods of flow cytometry, TBII, TSAB and TSBAB assays. Total T4 levels in the sera were measured using clinical total T4 human assay (Vitros® Eci Immunodiagnostic Systems, Ortho-clinical Diagnostics, Rochester, NY)

2.1. Flow Cytometry

CHO cells expressing full length human TSHR (JP19) was used. These adherent cells were detached using EDTA/EGTA (5mM each) in 1 x PBS. 150,000 cells/tube were transferred into Falcon 2052 tubes and washed in 3ml of 1 x PBS. Cells were pelleted at 500 x g, at 4^{o}C for 3min, and supernatant was removed by inversion. Cells were incubated for 30 min at room temperature in 100µl PBS-BSA 0.1% containing 5µl (5%) mouse serum. The cells were then washed in 3ml of 1 x PBS/0.1% BSA, centrifuged and supernatant removed as above, incubated for 30 min in the dark and on ice with 2µl fluorescein-conjugated γ-chain-specific goat anti-mouse IgG (Sigma Chemical Co., St. Louise, MO) in the same buffer. Propidium iodide (10µg/ml) was used for detection of damaged cells, which were excluded from the analysis. Cells were washed once again and supernatant removed as above and resuspended in final volume of 250µl in 1 x PBS/0.1% BSA. The fluorescence of 10,000 cells/tube was assayed by a FACScan flow cytometer (Becton Dickinson, Eerenbodegem, Belgium) [34].

2.2. TBII

TSH-binding inhibiting activity was also measured on JP19 cells [34]. Briefly, 5 x 10^4 cells/well were plated onto 96-well plates one day prior to experiment. Cells were incubated in 95µl of TBII Binding Buffer (5.4mM KCl, 0.44mM KH_2PO_4, 0.47mM $MgSO_4$, 0.35mM Na_2HPO_4, 1.3mM $CaCl_2$, 0.1% glucose, 9.5% sucrose, 5% BSA, pH 7.4), 30,000cpm $TSH^{I\text{-}125}$ and 5µl mouse serum/well (5%), for 4 hours at room temperature. At the end of the incubation period, the cells were rapidly rinsed twice with the same ice-cold buffer and finally solubilized with 0.2ml 1N NaOH before radioactivity was measured in a gamma counter. All

experiments were done in triplicate, and results are expressed as cpm bound. The stronger the TBII activity, the lower the cpm of bound $TSH^{I\text{-}125}$

2.3 TSAB and TSBAB

TSAB and TSBAB activities were measured using JP19 [34]. Briefly, 3 x 10^4 cells/well in 96-well plates were rinsed with Krebs-Ringer-HEPES (KRH) buffer (124mM NaCl, 5mM KCl, 0.25mM KH_2PO_4, 0.5mM $MgSO_4$, 0.4mM Na_2HPO_4, 1mM $CaCl_2$, 0.1% glucose, 20mM HEPES, and 0.3% BSA, pH 7.4) before being incubated in the same buffer, together with 25µM Rolipram and 5µl of serum in a total volume of 100µl/well. The cells were incubated for 4 hour at 37^0C. Cyclic AMP released into the medium was measured using a competitive binding assay kit (Perkin Elmer, Wellesley, MA). TSAB was measured under basal conditions described above while TSBAB was measured in identical condition, but with the addition of 10mIU/ml final concentration bovine TSH (Sigma Chemical Co. St. Louise, MO). Triplicate samples were assayed in all experiments. Commercial kits measuring cAMP were used (Perkin Elmer, Wellesley, MA) and results are expressed as pmol/ml. In measurement of TSBAB in sera, the higher the TSBAB activity level, the smaller the result in pmol/ml. In TSAB activity measurement in sera, the higher the activity, the higher the result in pmol/ml.

3. Cytokine Profile

Using Real-time PCR and TaqMan® probe techniques on cDNA reverse transcribed from RNA, gene transcription in spleen, thyroid and orbit were measured

to define the relative amount of Th1 (γIFN, IL2) and Th2 (IL4, IL5) cytokines present in immunized and non-immunized mice.

3.1. RNA Extraction

The area of the eye that was taken for RNA extraction was area 2 and 3 shown in figure 21 below. The weight of a normal eye of a mouse ranges from 14 to 24 mg [39]. It is technically difficult to distinguish orbital contents, such as fat from muscle, without compromising RNA integrity. For this reason, the entire content of the eye was enucleated and placed immediately in RNA Later^{TM} , a reagent used to preserve RNA in the tissue. Care was taken to prevent too much dissection and cutting of tissue to minimize RNase release into the tissue which can lead to RNA degradation.

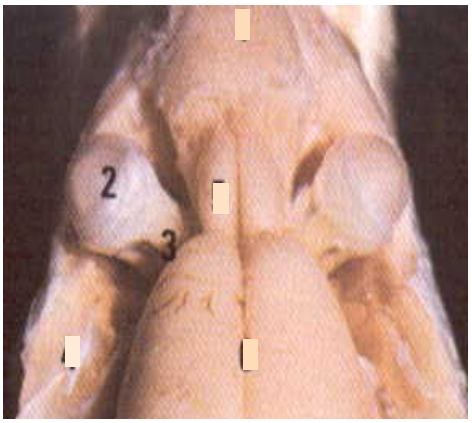


Figure 21: The eye of a rat *in situ* viewed from the top. [1]

Tissues obtained at sacrifice were first weighed before RNA was extracted following manufacturer's protocol with a few modifications. Briefly, 100mg tissue was homogenized in 1ml TRIZOL^{TM} (Invitrogen Corp, Carlsbad, CA), using PowerGen 125 (Fisher Scientific, Hampton, NH), passed through a 21G needle, centrifuged at 14,000g for 10 min at 4 ^{0}C to pellet DNA and non-homogenized tissue,

with final addition of 0.2ml Chloroform (Sigma Chemical Co. St. Louise, MO). The aqueous layer was pipetted into a Phase-Lock-Gel^{TM} (Eppendorf, Hamburg, Germany) tube and extraction was done using Acid Phenol (Ambion, Austin, TX). Phase-Lock-Gel^{TM} Tube was used for Acid Phenol extraction to minimize loss of aqueous phase. Subsequently, 2 Chloroform: Isoamyl Alcohol (24:1) (Sigma Chemical Co., St Louise, MA) extraction steps were done in normal 2.0 ml microfuge tubes with back extractions to maximize recovery of aqueous layer. Aqueous layer recovered from these phenol chloroform steps are DNA free because contamination from the DNA containing interphase layer was avoided. RNA was precipitated using equal volume of Isopropanol (Sigma Chemical Co., St Louise, MA) and 0.8M Disodium Citrate /1.2M Sodium Chloride (Sigma Chemical Co., St Louise, MA) and pelleted by centrifuging at 14,000g for 10 min at room temperature. RNA pellet was washed twice using 70% Ethanol (Sigma Chemical Co., St. Louise, MA) and air dried for 7 min before RNase free water was added. Extracted RNA was frozen overnight in minus 70^0C deep freezer. The next day, dissolved RNA sample was measured on spectrophotometer to determine the concentration and ran on 1% native agarose gel to check the integrity before proceeding to convert RNA to cDNA.

3.2. Reverse Transcription

RNA was reverse transcribed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), following manufacturer's protocol. Five µg of total RNA extracted was utilized for this conversion as shown in Table 2.

Table 2. Conversion of RNA to cDNA

Component	Volume µl/reaction
Total RNA + RNase DNase free H_20	8
Primer (50µM oligo dT)	1
10mM dNTP mix	1
Incubate at 65°C for 5 min, then place on ice for at least 1 min. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.	
10 x RT buffer	2
25 mM $MgCl_2$	4
0.1 M DTT	2
RNase OUT™ (40U/µl)	1
SuperScript™ III RT (200u/µl)	1
Add 10µl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.	
50 min at 50°C 5 min at 85°C	
Chill on ice. Collect the reactions by briefly centrifugation. Add 1µl of RNase H to each tube and incubate for 20 min at 37°C The resulting cDNA was directly used for Real-time PCR.	

3.3. Real-Time Polymerase Chain Reaction (PCR)

Real-Time PCR was carried out using the TaqMan® Gene Expression Assays (20x) (Applied Biosystems, Foster City, CA). This assay consisted of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB (minor groove binder) probe. Assays for genes IL2, IL4, IL5 and γIFN in *Mus musculus* were done. Each cytokine was multiplexed with β-actin, the housekeeping gene. These pre-designed gene specific Taqman® probes and primers which have been previously manufactured and passed quality control specifications, are proprietary designs owned by Applied Biosystems. These kits were used together with TaqMan® Fast Universal PCR Master Mix (2x) (Applied Biosystems, Foster City, CA) and ran on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each sample was prepared in a mixture detailed in table 3.

Table 3. Real-Time PCR Reaction Mix and Cycling condition

Components	Volume µl/reaction
Mouse β-actin Endogenous Control (20x)	0.25
TaqMan Gene Expression Assay (20x)	0.5
TaqMan Fast Universal Master Mix(2x)	5
cDNA template + dH_20	4.25
Cycling Conditions according to manufacturer's protocol 20sec 95^0C 40 cycles of 1 sec 95^0C 20 sec 60^0C	

Relative quantification using the comparative method was used to analyze the data output. To determine the change in cytokine expression after immunization with

TSHR, the $2^{-\Delta\Delta Ct}$ formula was applied. Samples from mice injected with TSHR were the experimental samples while samples from control mice served as calibrator samples. Results were reported as relative fold change of experimental sample over control sample. In determining relative Th1 or Th2 dominance, we calculated the fold changes were calculated using Th2 cytokines as the experimental samples with Th1 cytokines as the calibrator. A fold change of >1 meant that experimental samples had increase in expression over the calibrator samples while a fold change of <1 meant a decrease in expression.

4. Statistical Analyses

Data were analyzed and graphs plotted using SPSS version 9.0 software. Non-parametric data were expressed as median and inter-quartile ranges (25% to75%). Analyses of differences between groups were done using Mann-Whitney test. Linear regression equation was obtained using stepwise, enter method. Cross tabulation statistics were tested with Chi-square test. A p-value of <0.05 was taken as statistically significant.

IV. RESULTS

1. RNA Extraction

Absorbance readings and ratios for all tissue extracted were tabulated as shown in tables 4, 5,6,7,8 and 9. It is ideal that $A_{260/280}$ ratio be between 2.00 and 2.20 indicating clean RNA preparations.

Table 4. Absorbance readings for spleen RNA in Balb/c mice.

Spleen	mg	dilution	A_{260}	A_{280}	$A_{260/280}$	Conc.(mg/ml)
V1	107	100	0.704	0.323	2.178	5.65
V2	164	100	1.964	0.932	2.107	15.71
V3	115	100	0.931	0.432	2.154	7.45
V4	192	100	1.171	0.540	2.169	9.37
V5	155	100	1.053	0.490	2.150	8.43
2R1	105	100	0.623	0.281	2.220	4.98
2R2	131	100	0.875	0.399	2.192	7.00
2R3	111	100	0.925	0.423	2.188	7.40
2R4	100	100	0.603	0.272	2.216	4.83
2R5	65	100	0.650	0.300	2.180	5.21
3R1	108	100	0.675	0.305	2.214	5.40
3R2	82	100	0.990	0.450	2.210	7.97
3R3	53	100	0.740	0.330	2.210	5.93
3R4	76	100	0.820	0.370	2.210	6.53
3R5	128	100	0.830	0.380	2.220	10.00
4R1	160	200	0.670	0.300	2.235	5.36
4R2	130	200	1.026	0.467	2.199	8.21
4R4	142	400	0.868	0.410	2.118	13.88
4R5	154	200	0.965	0.450	2.143	7.72

Table 5. Absorbance readings for thyroid RNA in Balb/c mice.

Thyroid	mg	dilution	A_{260}	A_{280}	$A_{260/280}$	Conc.(mg/ml)
V1	58	100	0.424	0.187	2.260	1.69
V2	55	100	0.412	0.215	2.145	1.85
V3	72	200	0.353	0.156	2.260	1.41
V4	39	100	0.285	0.127	2.240	0.57
V5	109	100	1.436	0.669	2.146	5.74
2R1	136	100	0.870	0.396	2.196	3.48
2R2	39	50	0.554	0.255	2.177	1.11
2R3	47	50	0.847	0.389	2.180	1.69
2R4	80	200	0.385	0.315	2.179	5.48
2R5	47	50	0.726	0.338	2.147	1.45
3R1	48	100	0.518	0.236	2.192	2.07
3R2	40	50	0.448	0.205	2.192	0.90
3R3	60	100	0.991	0.460	2.156	3.96
3R4	92	200	0.868	0.397	2.187	6.94
3R5	34	50	0.550	0.252	2.184	1.10
4R1	83	100	0.590	0.270	2.186	2.39
4R2	104	100	0.696	0.311	2.241	2.79
4R4	102	100	1.048	0.474	2.210	4.19
4R5	30	50	0.353	0.156	2.263	0.71

Table 6. Absorbance readings for orbit RNA in Balb/c mice.

Orbit	mg	dilution	A_{260}	A_{280}	$A_{260/280}$	Conc.(mg/ml)
V1	47	50	0.809	0.366	2.207	1.62
V2	200	100	0.761	0.344	0.209	3.04
V3	80	50	0.833	0.377	2.209	1.67
V4	66	50	0.640	0.291	2.200	1.28
V5	49	50	0.650	0.299	2.173	1.30
2R1	67	50	0.669	0.366	2.185	1.34
2R2	55	50	0.644	0.290	2.217	1.29
2R3	61	50	0.685	0.310	2.206	1.37
2R4	86	50	0.864	0.394	2.195	1.73
2R5	74	50	0.721	0.326	2.208	1.44
3R1	78	50	0.653	0.290	2.255	1.31
3R2	77	50	0.614	0.275	2.234	1.23
3R3	61	50	0.450	0.193	2.330	0.90
3R4	61	50	0.654	0.289	2.265	1.31
3R5	54	50	0.745	0.334	2.233	1.49
4R1	59	50	0.641	0.283	2.262	1.28
4R2	78	50	0.748	0.328	2.278	1.50
4R4	84	100	0.438	0.186	2.353	1.75
4R5	103	100	0.443	0.187	2.373	1.77

Table 7. Absorbance readings for spleen RNA in Swiss Outbred mice.

Spleen	mg	dilution	A_{260}	A_{280}	$A_{260/280}$	Conc.(mg/ml)
V1	110	100	0.836	0.393	2.127	3.34
V2	54	100	1.606	0.712	2.164	6.43
V3	73	100	0.680	0.314	2.168	2.72
V4	98	100	1.070	0.497	2.155	4.28
V5	76	100	0.828	0.387	2.141	3.31
WT1	98	100	1.356	0.637	2.130	5.42
WT2	108	200	0.889	0.409	2.171	7.12
WT3	66	100	0.572	0.263	2.172	2.29
WT4	74	100	0.812	0.383	2.122	3.25
WT5	105	200	0.742	0.341	2.172	5.94
WT6	59	100	0.893	0.413	2.163	3.57
WT7	93	100	0.940	0.440	2.136	3.76
WT8	173	100	1.483	0.691	2.146	5.93
WT9	103	100	0.953	0.442	2.156	3.81
WT10	107	100	1.156	0.540	2.143	4.63

Table 8. Absorbance readings for thyroid RNA in Swiss Outbred mice.

Thyroid	mg	dilution	A_{260}	A_{280}	$A_{260/280}$	Conc.(mg/ml)
V1	3	50	0.153	0.071	2.142	0.31
V2	20	100	0.271	0.124	2.180	1.08
V3	11	100	0.076	0.034	2.226	0.30
V4	24	100	0.289	0.131	2.212	1.15
V5	42	100	0.352	0.162	2.173	1.41
WT1	39	100	0.393	0.179	2.197	1.57
WT2	53	100	0.395	0.181	2.180	1.58
WT3	41	100	0.324	0.147	2.200	1.30
WT4	42	100	0.700	0.323	2.167	2.80
WT5	35	100	0.138	0.052	2.645	0.55
WT6	28	100	0.178	0.066	2.714	0.71
WT7	67	100	0.211	0.079	2.668	0.84
WT8	25	100	0.206	0.080	2.564	0.82
WT9	41	100	0.290	0.137	2.120	1.16
WT10	42	100	0.448	0.194	2.307	1.74

Table 9. Absorbance readings for orbit RNA in Swiss Outbred mice.

Orbit	mg	dilution	A_{260}	A_{280}	$A_{260/280}$	Conc.(mg/ml)
V1	36	100	0.353	0.149	2.363	1.41
V2	41	100	0.345	0.150	2.303	1.38
V3	53	100	0.439	0.196	2.238	1.76
V4	38	100	0.447	0.206	2.166	1.79
V5	54	100	0.420	0.189	2.224	1.68
WT1	58	100	0.467	0.213	2.191	1.87
WT2	73	100	0.400	0.181	2.212	1.60
WT3	53	100	0.392	0.177	2.218	1.57
WT4	53	100	0.447	0.202	2.214	1.79
WT5	49	100	0.394	0.176	2.238	1.58
WT6	51	100	0.415	0.186	2.225	1.66
WT7	30	100	0.455	0.208	2.189	1.82
WT8	44	100	0.322	0.144	2.236	1.29
WT9	55	100	0.429	0.201	2.133	1.72
WT10	48	100	0.350	0.160	2.183	1.40

RNA integrity was check on native agarose gel (Figure 22).

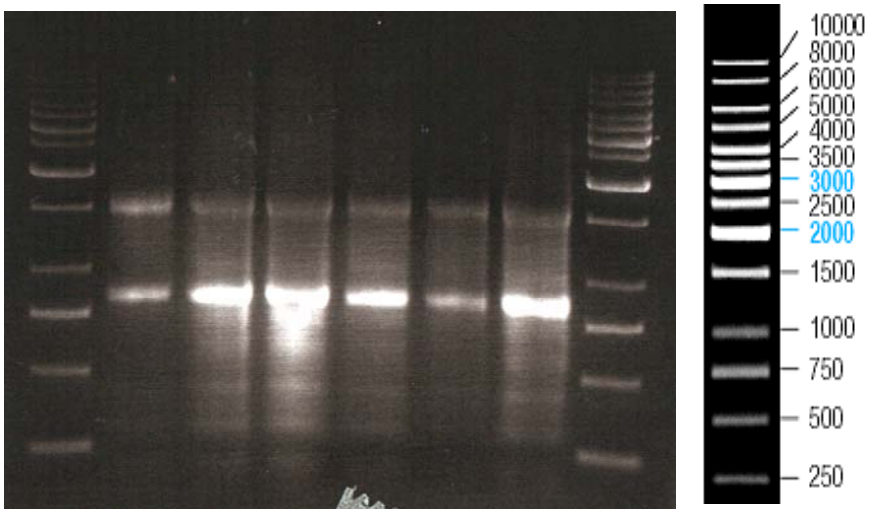


Figure 22. RNA on 1% native agarose gel.

First and last lanes are GeneRuler™ 1kb DNA Ladder (Fermentas, Ontario, Canada) with picture of bands and sizes shown. Lanes in between are Total RNA of 6 thyroid tissues.

2. Immunization of Balb/c and Swiss Outbred Mice

Mice were immunized with either an empty vector (control group) or vector containing TSHR expressing gene (treated group). Twenty Balb/c mice were injected, 5 with empty vector and 15 with TSHR. One mouse 4R3 died during the protocol before sacrifice. Five Swiss outbred mice were injected with empty vector and 10 were injected with TSHR making a total of 15 Swiss outbred mice injected. All results are expressed as median and interquartile ranges are in parentheses.

2.1 Weight

Weight changes in the two strains of mice are shown in Table 4. Control and treated mice were comparable at the beginning and at the end of the experiment (Figure 23). Both control and treated Swiss outbred mice showed increase in weight at the end of experiment ($p<0.05$), while Balb/c mice did not show any significant weight change during the protocol (table 10).

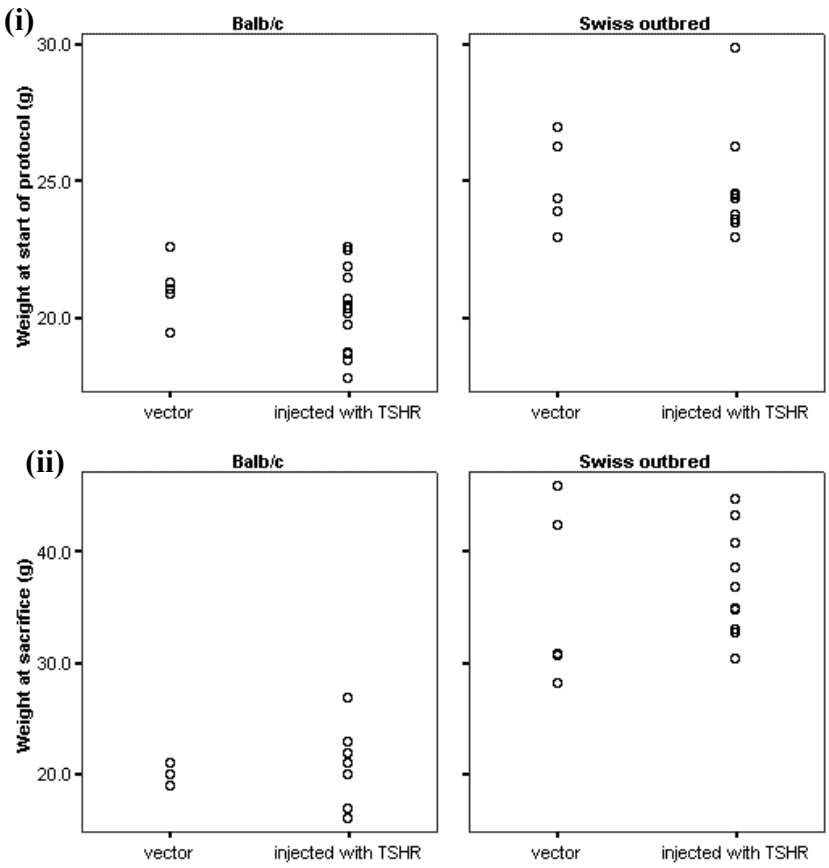


Figure 23. Weight of mice at the start and the end of protocol.

Weight in gram for control (vector) and treated (injected with TSHR) group at the (i) start of protocol and at (ii) sacrifice was shown.

Mice	Method	Weight at Beginning (g)	Significance of change (compared to control)	Weight at end (g)	Significance of change (compared to control)	Significance of change (from beginning to end of protocol)
Balb/c	Vector	21.1 (20.2 – 22.0)	-	20.0 (19.5 – 21.0)	-	Not Significant
Balb/c	TSHR	20.3 (18.8 – 21.6)	Not Significant	21.0 (16.8 – 22.3)	Not Significant	Not Significant
Outbred	Vector	24.4 (23.4 – 26.7)	-	30.9 (29.5 – 44.3)	-	<0.05
Outbred	TSHR	24.5 (23.6 – 26.3)	Not Significant	36.0 (33.0 –41.5)	Not Significant	<0.05

Table 10. Weight changes in Mice between control and treated group at beginning and end of experiment.

Results are expressed as median and interquartile ranges in parentheses.

2.2 Total T_4

There were no significant differences in Total T_4 level between the control and treated group of both the inbred and outbred strains (Table 11, Figure 24). Five Balb/c mice had Total T_4 above the range of Total T_4 recorded for control mice and 1 mouse had lowered total T_4 below this range.

In Swiss outbred mice, 2 mice had Total T_4 above the ranges for control mice and 3 had Total T_4 lower than the range.

Table 11. Total T_4 median for control and treated mice

Mice	Method	Total T_4 (pmol/L) at sacrifice	Significance of change (compared to control)
Balb/c	Vector	40.5 (23.9-43.8)	-
Balb/c	TSHR	57.0 (44.8-70.4)	Not Significant
Outbred	Vector	60.2 (56.1-84.7)	-
Outbred	TSHr	63.8 (59.2-75.6)	Not Significant

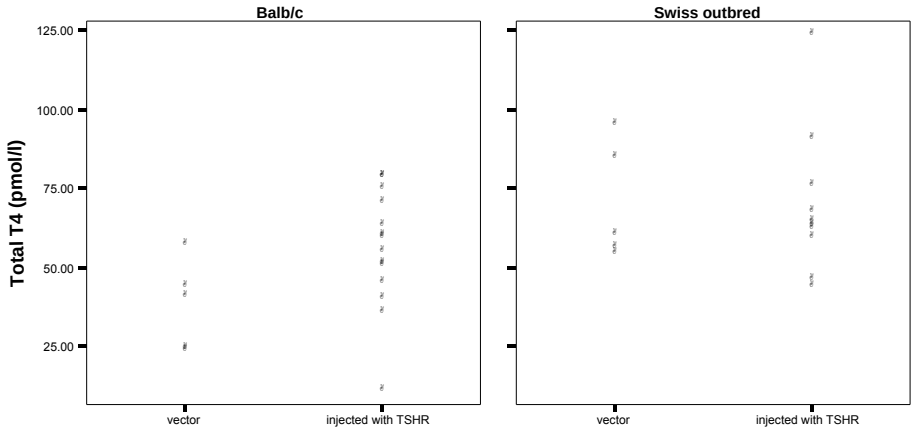


Figure 24. Total T_4 measurement in Balb/c and Swiss outbred

No significant difference between the control and treated group of both strains.

2.3 FACS for TRAB Detection

Most of the treated Swiss outbred mice contained autoantibodies which recognize native TSHR expressed on stable cell line, JP19. The median Fluorescence Unit of 37.4 in treated mice was compared to 7.2 in control mice was statistically significant. In contrast, there was no significant difference in the fluorescence unit between control and treated Balb/c mice (Table 12, Figure 25).

Table 12. FACS for control and treated mice

Mice	Method	Fluorescence Unit (FU) at sacrifice	Significance of change (compared to control)
Balb/c	Vector	6.1 (4.4 – 8.4)	-
Balb/c	TSHR	5.0 (4.2 – 8.9)	Not Significant
Outbred	Vector	7.2 (5.9 – 8.7)	-
Outbred	TSHr	37.4 (11.7 – 56.5)	<0.05

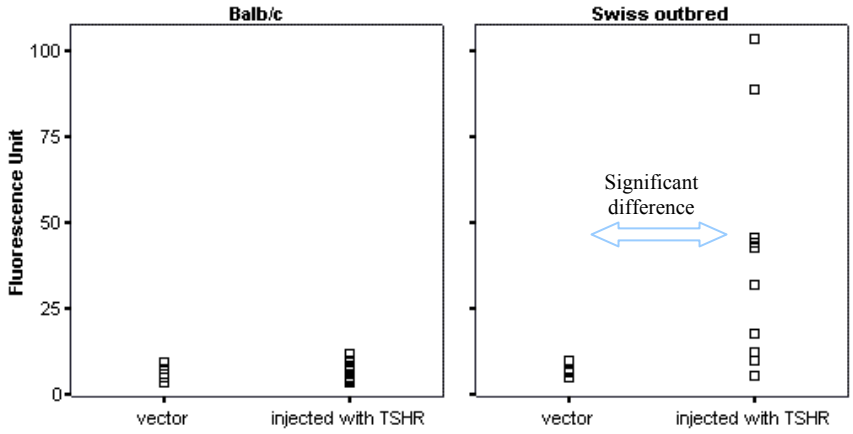


Figure 25. TRAB level detection using FACS.

Significant detection of TRAB level in treated Swiss outbred mice and no significant detection in treated Balb/c by flow cytometry.

2.4 TBII Assay

In Swiss outbred mice, a significant change (p<0.005) was observed between control and treated group. Sera of treated mice showed considerable TBII activity with a median of 74.5% (interquartile range of 45.9-85.9%), relative to median of 100% for controls (interquartile range of 97.2-100%) In Balb/c mice however, there was no significance difference between control and treated group.

Table 13. TBII Levels in control and treated mice.

Mice	Method	TBII (%) at sacrifice	Significance of change (compared to control)
Balb/c	Vector	86.0 (81.0 – 93.5)	-
Balb/c	TSHR	86.0 (76.0 – 97.5)	Not Significant
Outbred	Vector	100.0 (97.2 – 100.0)	-
Outbred	TSHr	74.5 (45.9 – 85.9)	<0.005

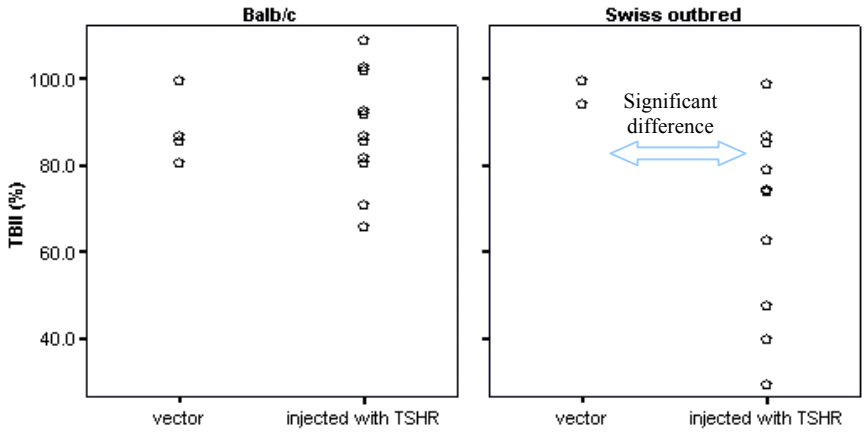


Figure 26. TBII activity in Balb/c and Swiss Outbred

No significant difference between control and treated groups of Balb/c. In Swiss Outbred, there was significance difference between the control and treated groups.

2.5 TSAB and TSBAB Bioassay

Results were calculated as percentage (%) of control average for both TSAB and TSBAB cAMP production. No significant difference in cAMP production was observed in the case of TSAB for all of Balb/c and Swiss Outbred mice (Table 14, Figure 27).

Table 14. TSAB activity in sera of Balb/c and Swiss Outbred

Mice	Method	TSAB (%) at sacrifice	Significance of change (compared to control)
Balb/c	Vector	67.1 (61.0 – 155.5)	-
Balb/c	TSHR	156.5 (50.8 – 291.7)	Not Significant
Outbred	Vector	87.7 (84.1 – 122.0)	-
Outbred	TSHr	82.5 (58.2 – 126.5)	Not Significant

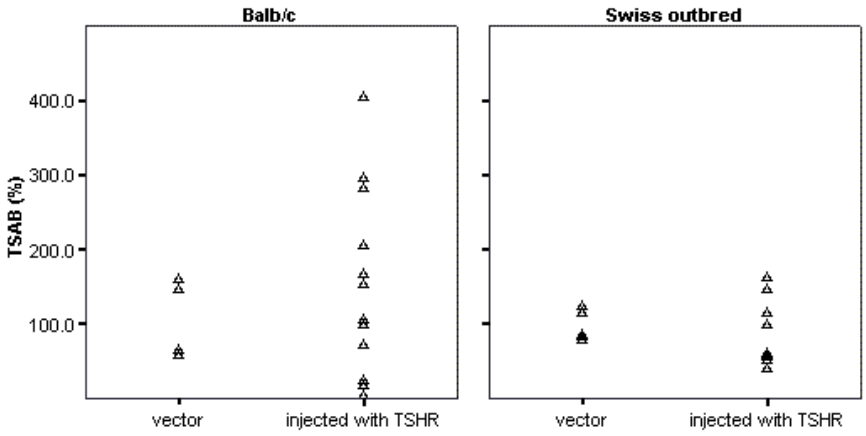


Figure 27. TSAB activity in Balb/c and Swiss Outbred

No significant difference between control and treated groups for both Balb/c and Swiss Outbred mice.

In the case of TSBAB, no significant difference between control and treated group of Balb/c mice was observed, while there was a significant difference ($p<0.05$) between control and treated group in the Swiss Outbred mice. In the treated group of Swiss Outbred, the median was 100.6% with an interquartile range of 81-111.3%, while the control group had a median of 136.2% with interquartile range of 112.2-139.7% (Table 15, Figure 28).

Table 15. TSBAB activity in sera of Balb/c and Swiss Outbred

Mice	Method	TSBAB (%) at sacrifice	Significance of change (compared to control)
Balb/c	Vector	104.2 (67.9 – 130.0)	-
Balb/c	TSHR	126.5 (17.5 – 135.8)	Not Significant
Outbred	Vector	136.2 (112.2 – 139.7)	-
Outbred	TSHr	100.6 (81.0 – 111.3)	<0.05

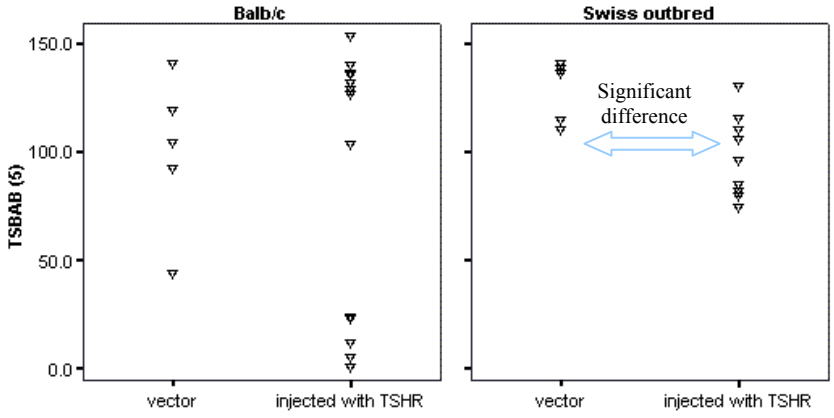


Figure 28. TSBAB activity in Balb/c and Swiss Outbred

No significant TSBAB activity between control and treated groups in Balb/c but in Swiss Outbred mice, there is significant difference between the control and treated group.

Cut off for TRAB measurements were set at > 2 Standard Deviations (SDs) away from mean of control mice. Table 16 showed cut off values for a parameter to be considered positive.

Table 16. Cut off values for the parameter to be considered positive

Balb/c	Positive if value is	Swiss outbred	Positive if value is
FACS	>10.8	FACS	>10.6
TBII	<71.4%	TBII	<93.9%
TSAB	>201.9%	TSAB	>141.2%
TSBAB	<27.5%	TSBAB	<98.8%

On FACS, only 1 was positive out of 14 treated Balb/c mice, while 8 out of 10 Swiss outbred were positive on FACS for autoantibodies recognizing TSHR (Table 17). TBII activity was present in 3 out of 14 Balb/c mice and in 9 out of 10 Swiss Outbred Mice, injected with TSHR (Table 18). The differences across strains were statistically significant. cAMP production detection for TSAB activity showed 5 of 13 positive Balb/c mice and 2 of 10 Swiss outbred mice positive with circulating TSAB (Table 19). TSBAB detection showed 5 out of 14 treated Balb/c group were positive while 5 out of 10 treated Swiss Outbred mice were positive (Table 20). The difference in TSAB and TSBAB were not significant across strains.

Mouse strain	FACS status		Total
	negative	Positive	
Balb/c	13	1	14
Swiss outbred	2	8	10
Total	15	9	24

Table 17. Cross tabulation of FACS status in the 2 strains of mice immunized with TSHR
p <0.005 (Significant)

Mouse strain	TBII status		Total
	negative	positive	
Balb/c	11	3	14
Swiss outbred	1	9	10
Total	12	12	24

Table 18. Cross tabulation of TBII status in the 2 strains of mice immunized with TSHR
$p < 0.005$ (Significant)

Mouse strain	TSAB status		Total
	negative	positive	
Balb/c	8	5	13
Swiss outbred	8	2	10
Total	16	7	24

Table 19. Cross tabulation of TSAB status in the 2 strains of mice immunized with TSHR
P >0.05 (Not Significant)

Mouse strain	TSBAB status		Total
	negative	positive	
Balb/c	9	5	14
Swiss outbred	5	5	10
Total	14	10	24

Table 20. Cross tabulation of TSBAB status in the 2 strains of mice immunized with TSHR
P >0.05 (Not Significant)

3. T cell Cytokine Profile using Real-Time PCR

Cytokine levels for spleen, thyroid and orbit were obtained using Real-time PCR and results expressed as fold difference between the treated and the control groups in both Balb/c and Swiss outbred (Table 21). In comparing the cytokine levels in the treated Balb/c and Swiss strain of mice, we found significant difference in expression in IL4 and IL5 in the spleen (p <0.05 and p <0.005 respectively) (Table 21, †), IL2 and γIFN in the thyroid (p<0.05 for both) (Table 21, †), IL4 and γIFN in the orbit (p<0.005) (Table 21, †).

Within each genetic strain, statistically higher expression of γIFN (in thyroid) and lower expression of IL5 (in orbit) were found in treated Balb/c mice (Table 21, **). In Swiss outbred, IL5 expression was higher in the spleen and thyroid of treated Swiss outbred compared to control. In contrast, expression of IL4, IL5 and γIFN were all lower in the orbit of TSHR injected (treated) Swiss outbred mice compared to control Swiss outbred mice (Table 21, **).

Tissue	cytokine	Balb/c Median	Swiss outbred Median	Significant difference, *P* (comparison of 2 strains)
Spleen	IL4	0.60 (0.51-0.84)	1.01 (0.73-1.23)	<0.05†
	IL2	1.30 (0.65-1.87)	0.87 (0.80-1.20)	Not Significant
	IL5	0.73 (0.55-1.10)	1.45 ** (1.16-1.57)	<0.005†
	γ IFN	1.81 (0.98-2.56)	1.06 (0.99-1.42)	Not Significant
Thyroid	IL4	0.63 (0.26-0.88)	0.58 (0.40-1.04)	Not Significant
	IL2	1.63 (0.74-2.43)	0.45 (0.17-0.88)	<0.05†
	IL5	0.61 (0.44-1.03)	1.81** (1.38-2.73)	Not Significant
	γ IFN	3.53** (1.40-4.37)	1.37 (0.75-1.75)	<0.05†
Orbit	IL4	1.22 (1.03-2.60)	0.35** (0.30-0.84)	<0.005†
	IL2	1.12 (0.59-2.68)	0.53 (0.33-0.92)	Not Significant
	IL5	0.71** (0.49-0.92)	0.60** (0.56-0.91)	Not Significant
	γ IFN	1.16 (0.96-3.21)	0.57** (0.44-0.83)	<0.005†

Table 21. Relative fold change of cytokine in Balb/c and Swiss outbred mice injected with TSHR compared to controls

**Significant difference ($p < 0.05$) between TSHR injected and control Balb/c mice.
**Significant difference ($p < 0.05$) between TSHR injected and control Swiss mice.
† Significant difference (<0.05) between Balb/c and Swiss outbred mice in spleen.
† Significant difference (<0.05) between Balb/c and Swiss outbred mice in thyroid.
† Significant difference (<0.05) between Balb/c and Swiss outbred mice in the orbit.
Interquartile range in parentheses.

3.1. Correlation of Cytokines with TRAB Measurements in Balb/c Mice

In spleen of treated Balb/c mice, there is correlation between γIFN and TSBAB with $p<0.05$. The stronger the TSBAB activity (the smaller the value), the greater the fold increase for γIFN (Figure 29). Correlation between IL2 and TBII was significant where a decline in IL2 fold change was observed with TBII activity increase (smaller value with increase TBII activity) (Figure 30).

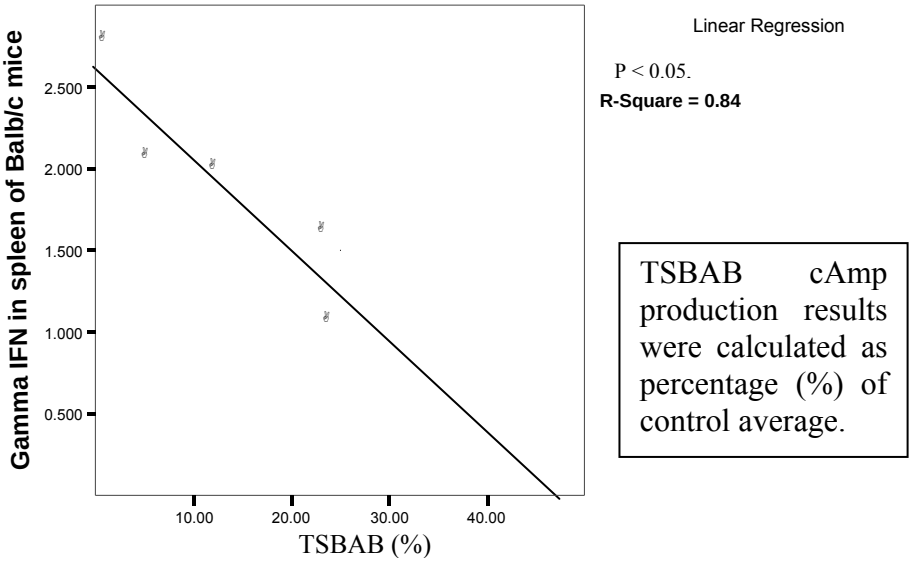


Figure 29. Correlation between γ IFN and TSBAB in spleen of Balb/c.

γ-IFN correlated with TSBAB in mice positive for blocking antibodies ($p<0.05$)
The stronger the TSBAB, the higher the fold rise in γ-IFN

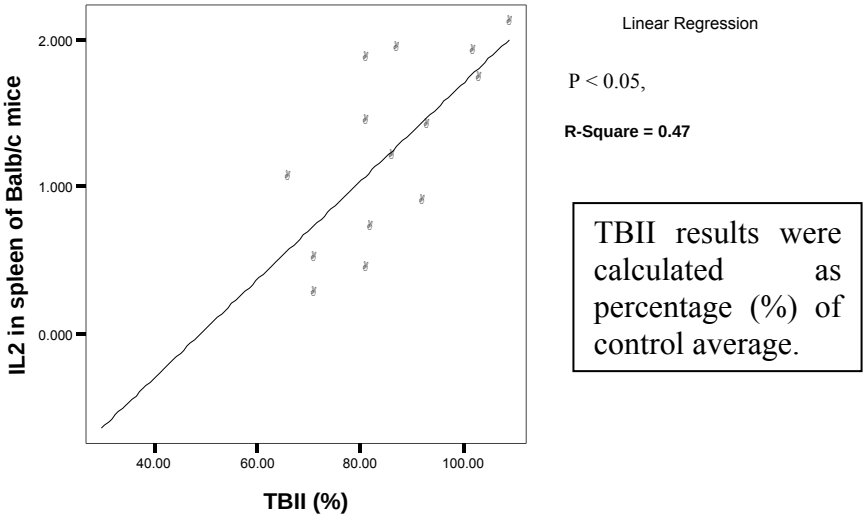


Figure 30. Correlation between IL2 and TBII in spleen of Balb/c.

IL2 correlated with TBII with a p value of <0.05. The stronger the TBII, the fold change in IL2 decline.

In the orbit, correlation between IL2 and TSBAB was significant (p<0.05). The stronger the TSBAB activity, the greater the fold increase in IL2 (Figure 31). Likewise for γIFN, the stronger the TSBAB activity, the higher the fold rise for γIFN (Figure 32).

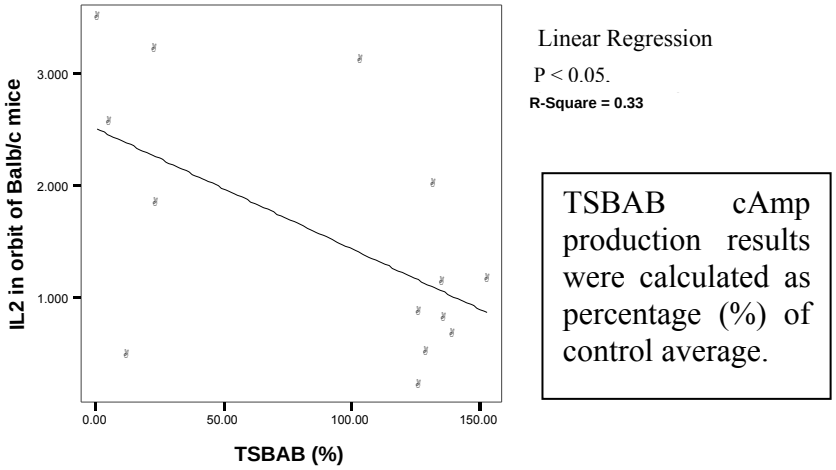


Figure 31. Correlation between IL2 and TSBAB in orbit of Balb/c.

IL2 correlated with TSBAB (p<0.05). The stronger the TSBAB, the higher the fold rise in IL2.

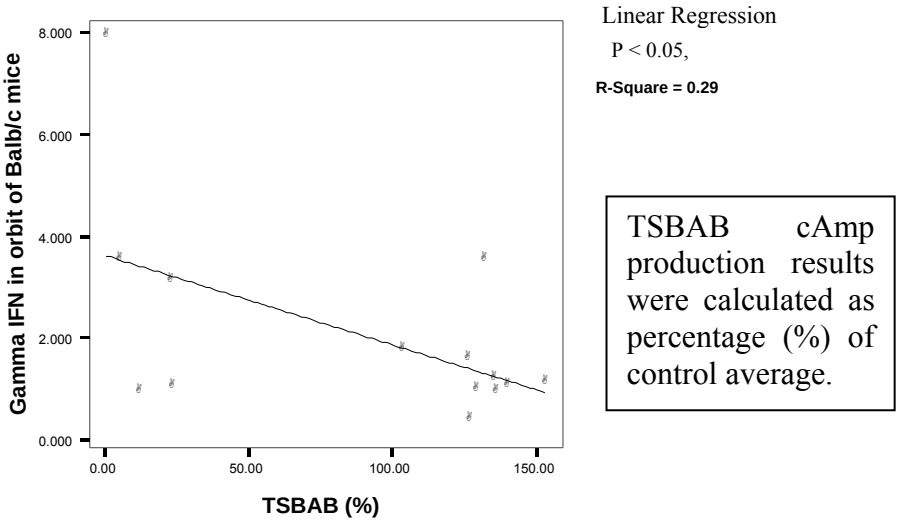
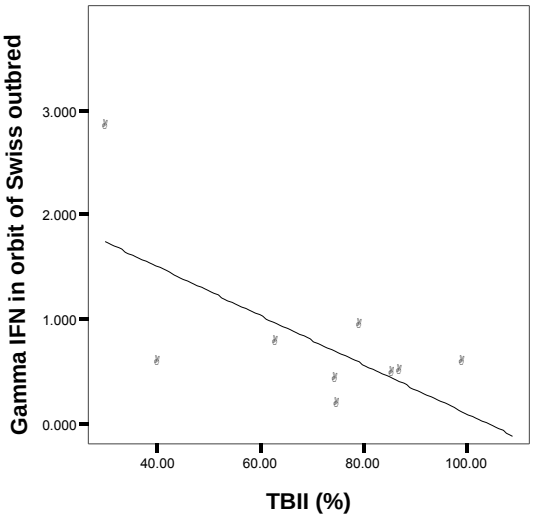


Figure 32. Correlation between γ IFN and TSBAB in orbit of Balb/c.

γ-IFN correlated with TSBAB (p<0.05). The stronger the TSBAB, the higher the fold rise in γ-IFN.

3.2. Correlation of Cytokines with TRAB Measurements in Swiss Outbred Mice

In the orbit of Swiss outbred mice, there is significant correlation between γIFN and TBII, where the stronger the TBII activity, the higher the fold rise in γIFN (Figure 33).



Linear Regression
P < 0.05
R-Square = 0.45

TBII results were calculated as percentage (%) of control average.

Figure 33. Correlation between γ IFN and TBII in orbit of Swiss outbred.

γ-IFN correlated with TBII ($p<0.05$). The stronger the TBII, the higher the fold rise in γ-IFN.

In thyroid of treated Swiss outbred mice, there is correlation, though the change did not reach statistical significance (p=0.05), between IL2 and TBII activity. The stronger the TBII activity (meaning the smaller the %), the higher the fold change for IL2.

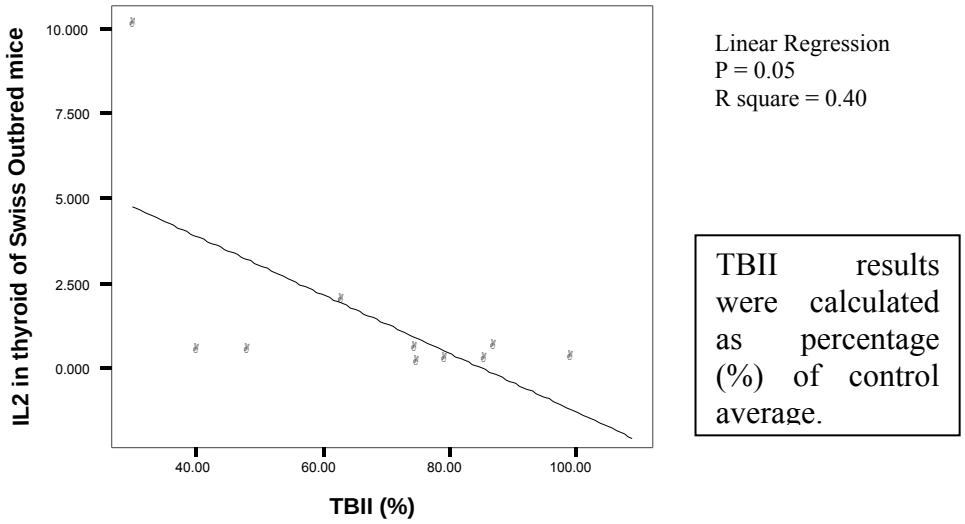


Figure 34. Correlation between IL2 and TBII in thyroid of Swiss mice

IL2 varied with TBII but the change did not reach statistical significance (p=0.05)

In the spleen of Swiss outbred mice, there is significant correlation ($p<0.05$) between IL5 and FACS. The correlation is and inverse correlation, where stronger the FACS signal, the lower the fold change in IL5 (Figure 35). However, if only FACS positive mice were included, the correlation became stronger ($p<0.005$) (Figure 36).

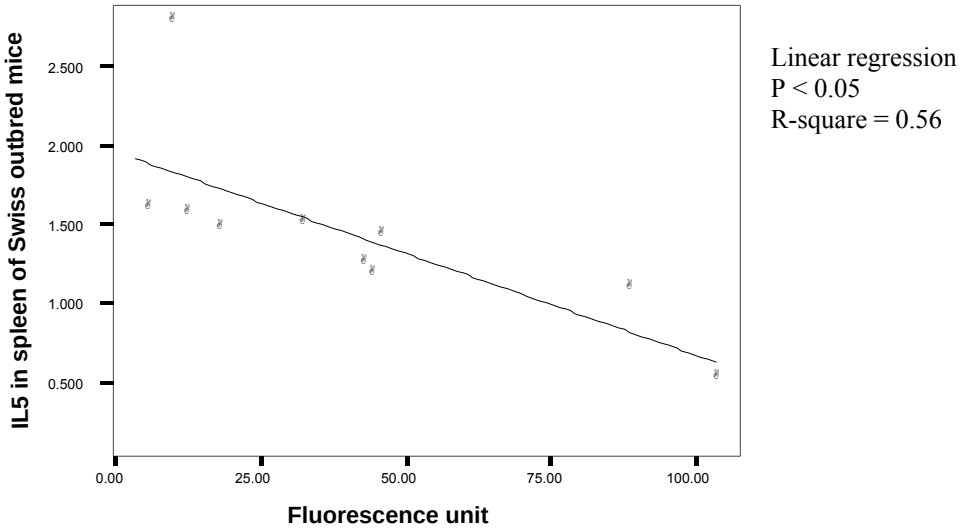


Figure 35. Correlation between IL5 and FACS in spleen of Swiss outbred.

IL5 correlated inversely with FACS ($p<0.05$). The stronger the FACS signal, the lower fold change of IL5

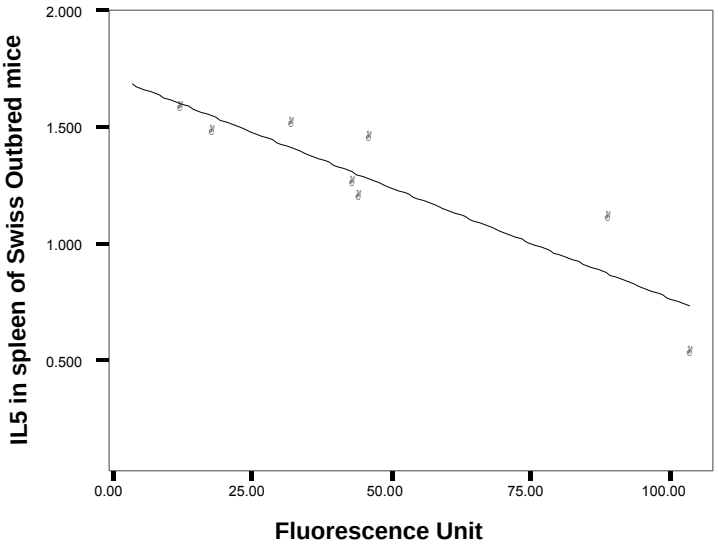


Figure 36. Correlation between IL5 and FACS in spleen of Swiss outbred.

IL5 correlated inversely with only FACS positive mice ($p<0.005$).

There is also correlation between IL5 and TSBAB in the spleen of Swiss outbred mice. The stronger the TSBAB activity, the larger the fold rise in IL5 with a p value of <0.05.

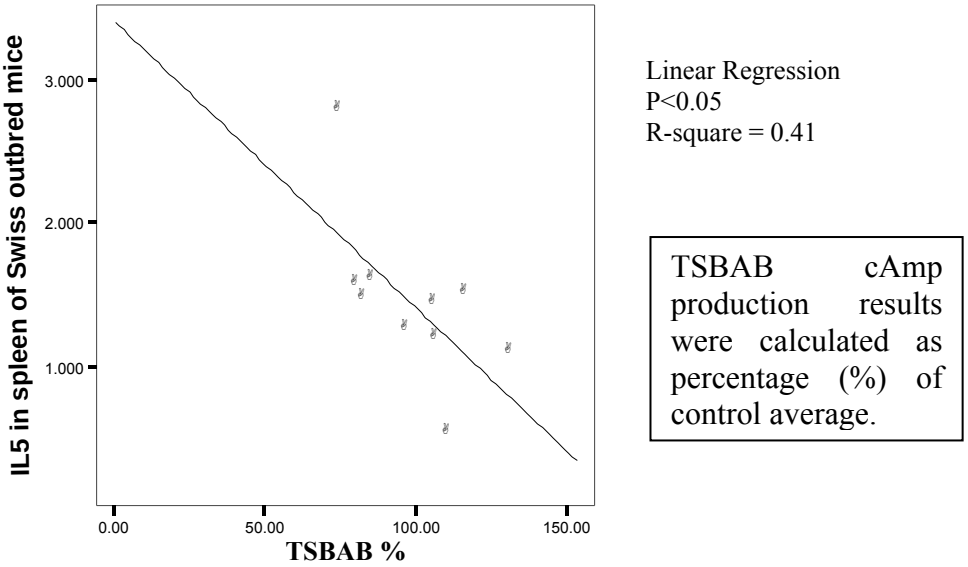


Figure 37. Correlation between IL5 and TSBAB in spleen of Swiss mice.

IL5 is correlated with TSBAB ($p<0.05$). The stronger the TSBAB, the larger the fold rise in IL5

The ratio of Th2 cytokines to Th1 cytokines in spleen, thyroid and orbit of Balb/c and Swiss outbred as is shown in table 22. There were significant differences between the two strains of mice in Th2 to Th1 ratio. They are indicated in the last column of table 22. Differences were observed between the two strains with respect to IL5:IL2, IL4:γIFN in the spleen; IL5:γIFN and IL5:IL2 in the thyroid and IL5:γIFN, IL5:IL2 and IL4:IL2 in the orbit.

Tissue	Th2:Th1	Balb/c Median	Swiss outbred Median	Significant difference, *P* (comparison of 2 strains)
Spleen	IL5: γIFN	0.027 (0.019-0.034)	0.022 (0.019-0.032)	Not Significant
	IL5:IL2	0.029 (0.024-0.041)	0.083 (0.052-0.104)	<0.005
	IL4:IL2	2.07 (1.29-2.71)	3.30 (2.45-4.47)	<0.05
	IL4:γIFN	1.52 (1.15-2.43)	0.97 (0.69-1.21)	<0.005
Thyroid	IL5: γIFN	0.22 (0.11-0.53)	89.39 (22.4-283.61)	<0.005
	IL5:IL2	0.10 (0.05-0.40)	219.70 (25.89-792.26)	<0.005
	IL4:IL2	1.35 (0.61-3.24)	3.25 (0.70-5.07)	Not Significant
	IL4:γIFN	2.31 (0.90-3.83)	1.57 (1.06-2.40)	Not Significant
Orbit	IL5: γIFN	15.90 (5.97-23.12)	6.14 (4.50-10.02)	<0.05
	IL5:IL2	9.02 (6.40-19.07)	3.22 (1.68-5.30)	<0.005
	IL4:IL2	0.30 (0.26-0.50)	0.18 (0.15-0.26)	<0.05
	IL4:γIFN	0.43 (0.23-0.71)	0.48 (0.16-0.92)	Not Significant

Table 22. Relative fold change of Th2 cytokines to Th1 cytokines in mice injected with TSHR plasmids.

Interquartile range in parentheses.

4. Summary of Findings

4.1. Changes after Genetic Immunization

There was no significant change in total T4 levels between mice injected with receptor or empty plasmids in the outbred and inbred mouse strains. Swiss outbred mice however put on more weight upon completion of protocol and this arose independent of T4 changes. The described method of immunization produced significantly higher numbers of mice positive for FACS and TBII assays in Swiss outbred compared to Balb/c (Table 12 and 13). The titers of these 2 assays were also significantly higher in outbred than in inbred mice. The numbers of animals positive for TSAB or TSBAB in the 2 mouse strains were similar although the absolute TSBAB level in Swiss outbred mice injected with receptor were significantly stronger than those receiving empty plasmids (Table 14 and 15).

4.2. Th1 and Th2 Cytokine Expression

The cytokine profiles in mouse models of GD were studied and differences in cytokine gene expression in various tissues between Balb/c and Swiss outbred mice were demonstrated. Table 23 summarizes these findings.

1. In the spleen,
    - Balb/c mice injected with human TSHR have γIFN, IL2, IL4 and IL5 expression levels comparable to that found in controls. In Swiss outbred mice, expression of IL5 (Th2) was found to be significantly higher than that in controls ($p<0.05$). Across strains of treated mice, the fold increase of IL5 in Swiss outbred and the fold drop in IL4 in Balb/c was statistically significant (Table 21, †).

- Within each individual Balb/c or Swiss outbred mouse injected with human TSHR, expression of IL5 (Th2) was much lower relative to Th1 cytokines γIFN and IL2. In contrast, expression of IL4 (Th2) was higher relative to these Th1 cytokines (Table 22).
- In Balb/c mice, the higher the intensity of antibody production as measured by TSBAB, the higher the γIFN (Th1) expression (Figure 29). This is in contrast to IL2 (Th1), which declined with intensity of TBII (Figure 30). In Swiss outbred mice, the higher the intensity of antibody production as measured by TSBAB, the higher the IL5 (Th2) expression (Figure 37). This is in contrast to the relationship of IL5 to FACS, which was inverse; i.e. the higher the FACS, the lower the IL5 (Figure 35 and 36). It would appear that the expression of various cytokines correlated differently with different measurements of TRAB production.

2. In the thyroid,
   - Balb/c mice injected with human TSHR have expression of Th1 cytokine γIFN was higher in compared to controls ($p<0.005$) (Table 21, **). This rise of γIFN in Balb/c was significantly higher than in Swiss outbred mice (Table 21,†). In contrast, the expression of Th2 cytokine IL5 was higher in Swiss outbred mice injected with human TSHR than controls ($p<0.05$) (Table 21, **). The drop in IL2 in Swiss outbred is significant compared to the change in Balb/c mice (Table 21,†).
   - Within each individual Balb/c mouse injected with human TSHR, expressions of IL5 (Th2) to γIFN or IL2 (Th1) cytokines were consistently lower (Table 22). In contrast, in Swiss outbred mice injected with the

receptor, expression of IL5 (Th2) was markedly enhanced compared to either γIFN or IL2 (Th1). These differences between the 2 mouse strains were statistically significant (Table 22).

- Although there was a trend for increasing TBII response to increasing level of IL2 in Swiss outbred mice, this did not reach statistical significance (Figure 34). The rest of the TRAB measurement did not correlate with the cytokine expression.

3. In the orbit,

   - Balb/c mice injected with TSHR, have significantly lower expression of IL5 than those exposed to empty plasmid ($p<0.05$) (Table 21, **). Expressions of γIFN (Th1) and expressions of IL4 and IL5 (Th2) were also all significantly lower in Swiss outbred mice injected with the receptor compared to the controls ($p<0.05$) (Table 21, **). The lowering of IL4 and γIFN were significant compared to the changes seen in Balb/c mice (Table 21. †).
   - Within each individual Balb/c or Swiss outbred mouse injected with human TSHR, the expression of IL5 (Th2) relative to the Th1 cytokines (IL2 and γIFN) were higher whilst that of IL4 (Th2) relative to IL2 or γIFN were lower (Table 22).
   - In Balb/c mice, the higher the TSBAB production, the higher the rise in IL2 and γIFN (Th1) (Figure 31 and 32). In Swiss outbred mice, γIFN observed similar trend with TBII measurements (Figure 33).

	Spleen		Thyroid		Orbit	
After treatment, Balb/c vs Swiss outbred	For IL5 - Balb/c < Swiss outbred For IL4 - Balb/c > Swiss outbred		For γIFN - Balb/c > Swiss outbred For IL2 - Balb/c > Swiss outbred		For IL4 - Balb/c > Swiss outbred For γIFN - Balb/c > Swiss outbred	
	Balb/c	Swiss	Balb/c	Swiss	Balb/c	Swiss
Control (C) vs Treated (T)	comparable	For IL5 - C < T	For γIFN – C < T	For IL5 - C < T	For IL5 - C > T	For γIFN - C > T For IL4 - C > T For IL5 - C > T
Th2 vs Th1	IL5 < γIFN & IL2 IL4 > γIFN & IL2 (Th2) (Th1)	IL5 < γIFN & IL2 IL4 > γIFN & IL2 (Th2) (Th1)	IL5 < γIFN & IL2 (Th2) (Th1)	IL5 > γIFN & IL2 (Th2) (Th1)	IL5 > γIFN & IL2 IL4 < γIFN & IL2 (Th2) (Th1)	IL5 > γIFN & IL2 IL4 < γIFN & IL2 (Th2) (Th1)
TRAB vs cytokine	↑TSBAB with ↑γIFN ↑TBII with ↓IL2	↑TSBAB with ↑IL5 ↑FACS with ↓IL2	No correlation	No correlation	↑TSBAB with ↑IL2 ↑TSBAB with ↑γIFN	↑TBII with ↑γIFN

Table 23. Summary of cytokine profile and immunological markers in control and treated groups of Balb/c and Swiss outbred mice.

V. DISCUSSION

1. Technical Difficulties

During the course of this study, a few technical difficulties were encountered.

- Sera samples taken from tail vein of mice at the start of the protocol posed a few challenges. First of all, collecting blood from tail vein was time consuming. With each passing minute of blood collection from the tail vein, the body temperature of the mouse being bled decreased, causing the tail vein to constrict, therefore, even less blood could be collected before the flow stopped altogether. Secondly, drops of blood that were collected using capillary tubes would often clot if the blood flow was slow. Thirdly, sera collected were often lysed due to its contact with skin on the tail. This might have affected the biochemical results which relied on color for measurement. Collection of sera at sacrifice by cardiac puncture though less time consuming and simpler, still run the same risk of being lysed when the syringe that was used for collection went right through the heart as happened with a few sera sample.
- Due to the small volume of blood allowed for collection without killing the mice at the start of the protocol, sera sample obtained were often limited (<60 µl). Therefore, the volume used for biochemical and immunological marker testing were kept at the smallest volume allowed without compromising the accuracy of the tests. There was no room for mistakes for because for some samples, there weren't enough sera for experimental repetition for certain samples.
- RNA extraction protocol was long and tedious due to the small specimen size. Much care had to be taken when separating the aqueous layer which contained the

RNA, from the interphase and organic layer which contained DNA and protein in order to obtain a DNA free RNA preparation. Due to the small specimen size, especially for the thyroid specimens, RNA yield was also low. When RNA yield is low, cDNA used for Real-Time PCR was low in concentration causing the expression of cytokines of interest to be undetectable. These were the case in 1 orbit sample and 2 thyroid samples in Swiss outbred and Balb/c strain respectively.

- Real-Time PCR for cytokines generated unexpectedly low expression. The probes and primers used initially were not sensitive and specific enough an accurate reading. There were inconsistencies even within the triplicate sample. When the expression of a gene is very low, coupled with non ideal primers and probes, the difference of one copy of gene of interest can be translated into a Ct value change of 2 – 3, therefore, generating inconsistent results within the same sample. After wasting much time and money trying to overcome these problems, new sets of primers and probes from Applied Biosystems, (TaqMan® Gene Expression Assays), which were tested and proven sensitive and specific were used in replace of the inferior primers and probes. The machine used for amplification was also switched to accommodate the new primer and probe sets.

2. Discussion of results

2.1. Genetic Immunization in Balb/c versus Swiss Outbred mice

Genetic immunization appeared to work better in Swiss outbred than Balb/c mice. It produced more FACS- and TBII- positive outbred mice. It also generated stronger TSBAB levels which correlated significantly with specific cytokine in specified tissue compartment. Specifically, it had a linear relationship with the expression of Th1 cytokines i.e. γIFN in the spleens & orbits and IL2 in the orbits of Balb/c mice. In Swiss outbred, TSBAB had a linear relationship with the expression of Th2 cytokine IL5 of the spleens. Therefore, TSBAB appeared to be a surrogate marker for Th1 cytokine in Balb/c and Th2 cytokine in outbred mice.

Amongst the cytokines, γIFN was more abundant in the thyroid in treated Balb/c mice (Table 21, **) whereas IL5 was more abundant in the thyroid and spleen of treated Swiss outbred mice (Table 21, **). Striking differences were also present amongst the 2 mouse strains in the IL5: γIFN and IL5: IL2 ratios within the thyroid, where the ratios in Balb/c indicated a γIFN and IL2 dominance, whereas the ratios in Swiss outbred showed a IL5 dominance (Table 22). Thus there appeared to be a bias towards a Th1 response in Balb/c and Th2 response in Swiss outbred in thyroid and spleen. The exception is in the orbit where in both mouse strains, IL5 expression was lower compared with controls (Table 21). In Swiss outbred, other cytokine such as IL4 and γIFN were also reduced in TSHR injected mice in comparison with controls (Table 21). Thus in orbits of Swiss outbred mice, both Th1 and Th2 cytokines were down regulated where as in inbred Balb/c only IL5 was affected.

2.1.1. Genetic Immunization findings in Balb/c Mice

Genetic immunization was also efficient in generating mAbs against the TSHR. Genetic immunization with TSHR cDNA in Balb/c mice was first described in 1998 by Costagliola et al [34]. Signs of thyroiditis were observed in the 14 immunized Balb/c mice. Clusters of immune cells were found at the periphery of the thyroid and they comprised $CD4^+$ T cells and B cells. With a single exception, 19 of the 20 mice immunized with the expression cDNA construct developed anti-TSHR antibodies. Most of these antisera recognized both a recombinant fusion protein made in *E. coli* and the native TSHR expressed at the surface of CHO cells. Most of the antisera presented a TBII or TSBAB activity, and one serum exhibited a clear and specific TSAB activity although this mouse had normal T4 level. Whereas TBII and TSBAB activities can be easily obtained by immunization against recombinant immunogens, TSAB activity had been hard to obtain with earlier protocols. In contrast, similar technique done in Balb/c mice produced very different results in the hands of another group of researchers. Pichurin P et. al. reported absence of lymphocytic infiltration in the thyroids regardless of whether the mice did or did not have TRAB [40]. TRAB were undetectable in all DNA vaccinated Balb/c although in some animals, low levels can be found by flow cytometry. The most recent publication on this subject by Baker G et al showed that genetic immunization was able to elicit TSAB and TBII response despite absence of histological evidence of thyroiditis [41]. Therefore, antibody responses towards a specific method of vaccination can vary despite experimentation in the same mouse strain depending on research groups. The findings in the cohort of Balb/c mice were closer to the results reported by Pichurin P et al in that only 1 out of 14 mice were FACS positive and 3 out of 14 were TBII positive and their titers were low. However, generation of

TRAB with functional characteristics was more successful compared to the groups of Pichurin and Costagliola because there were 5 mice positive for TSAB and 5 mice positive for TSBAB.

One can only speculate about the basis for these differences. The same mouse strain and the same immunization protocol were used. Cardiotoxin, the agent found by Costagliola *et al.* to provide the strongest enhancement of genetic immunization was also given. One factor could be the differing origins of the mice. The animals were derived from different breeding colonies, Charles River in the study by Costagliola *et al.*, Jackson Laboratory in the study by Pichurin et al and in our case Animal Holding Unit (AHU), MD1, National University of Singapore. Thus differences in breeding protocols, environmental factors and housing conditions may affect results. Facilities used by a number of researchers (apparently including that used by Costagliola *et al.*) are not pathogen-free. Those used by Pichurin et al and the local animal facilities used in my study are in contrast pathogen-free. Ironically, this may be less suited for the development of an animal model resembling Graves' disease in humans. Infections have been known to play a part in the pathogenesis of autoimmune thyroid disease. It is possible therefore, that infections could contribute to the expression of experimentally induced Graves' disease. Strong evidence for environmental influences on immunological response also came recently from a report by Baker G et al where in a transfer model, antigen-induced secretion of IL4 from splenocytes of recipient animals varied between the Cardiff and Brussel animal holding facilities despite the using the same primed splenocytes or lymphocytes.

In the study by Pichuria et al, low or undetectable levels of TSHR antibody in DNA vaccinated mice did not preclude the possibility of lymphocyte sensitization to the TSHR [40]. Indeed, splenocytes from TSHR-DNA vaccinated mice proliferated

and produced large amounts of γIFN in response to challenge with purified TSHR-289, an ectodomain variant corresponding to the TSHR A-subunit [40]. Splenocyte proliferation and γIFN production were unrelated to TSHR antibodies in the donor mouse's serum. In addition, the genetic immunization study by Costagliola et al produced 3 monoclonal antibodies of IgG2a-κ isotype [34]. The IgG2a- κ isotype reflects a switch to Th1, probably as a consequence of γIFN production. The findings in my study were corroborated by the above mentioned data. γIFN was found to be more abundant in the thyroids of TSHR vaccinated Balb/c mice than those of controls. There was a dominance of γIFN and IL2 to IL5 in the ratio calculation of the thyroidal cytokines. TSBAB also had a linear relationship with the expression of Th1 cytokines i.e. γIFN in the spleens & orbits and IL2 in the orbits of Balb/c mice.

Finding of γIFN production in TSHR-vaccinated Balb/c mice is unusual because this strain of mouse typically shows a bias toward Th2 responses, at least with respect to Leishmania infection [42]. Moreover, it was previously shown by Many et al (a collaborator of Costagliola) that TSHR-primed T cells generated by DNA vaccination and transferred to naive Balb/c recipients induced thyroiditis characterized by a Th2 type response, with cells containing IL-4 and IL-10 [33]. Yet, my current data is consistent with numerous other observations that DNA vaccination against diverse antigens induces Th1-type responses as reflected in γIFN production and antibodies of subclass IgG2a [43-45].

2.1.2. Genetic Immunization findings in Swiss Outbred Mice

Genetic immunization of outbred NMRI mice was also first reported by Costagliola et al [32] in which all animals had antibodies detected by flow cytometry and 1 out of 5 female outbred mice developed T4 elevation with circulating TSAB

accompanied by thyroid hyperplasia. Their glands displayed a lymphocytic infiltration characteristic of a Th2 reaction with IL4 producing T cells and B cells. A great majority (~75%) developed blocking TSBAB. The TRAB profiles found in my cohort of experimental Swiss outbred mice were similar with regards to flow cytometry (8 positive out of 10) and TBII (9 positive out of 10). Development of TSAB was also detected in 2 mice out of cohort of 10 animals although only one of the 2 had elevated total T4 level. TSBAB was only found in 50% of the animals. Expression of Th2 cytokine IL5 was higher in Swiss outbred mice injected with TSHR compared to controls in the splenic and thyroidal tissues. There was also a drop in expression of IL2 (Th1) cytokine in the vaccinated thyroid relative to control, which differ significantly from that in Balb/c mice. There was also a large dominance of IL5 to IL2 or γIFN expression in the ratio calculation and this contrast sharply with the findings in Balb/c mice. Thus Swiss outbred mice tended towards a Th2 bias upon genetic immunization with the human TSHR. Correlation of cytokines with various TRAB measurements tended to be heterogeneous with no consistent direction of change. Finding of Th2 response is not surprising since due to the role of autoantibodies, Graves' disease has long been considered to be a Th2-dominated disease. Support for this hypothesis includes features of atopy such as elevated serum IgE in some Graves' patients [46] as well as the induction of Graves' disease in patients with multiple sclerosis (a Th1-dominated autoimmune disease) following treatment with monoclonal anti-CD52 antibody [47]. On the other hand, cytokine expression profiles in sera and thyroid tissues from Graves' patients indicate a mixed Th1/Th2 immune response [48]. Moreover, Graves' disease generally ameliorates during pregnancy, a Th2-dominant state [49]. Even more important, TSAB are IgG1 [13] a Th1-type subclass in humans.

A model of GO had been described by transfer of TSHR-primed T cells to naive syngeneic recipients where priming was achieved by genetic immunization (GI) with a plasmid encoding the human TSHR. Th2 thyroiditis developed in ~ 60% of the recipients, about one third of these animal with thyroiditis also displayed orbital changes, including immune infiltration and edema, similar to those seen in GO [33]. The severity of the orbital changes correlated positively with the extent of the Th2 skew in the thyroid immune infiltrate of that animal. GO changes can also be observed by genetic immunization of NMRI outbred mice. Orbital changes include extraocular muscles dissected by edema and a fibrotic amorphous material accompanied by macrophages and mast cells in approximately 20% of the animals [32]. However, ocular muscle anatomy is complex and easily disturbed if muscle contraction occurs before fixation. Recent work by Baker et al suggested that histological GO findings described by Many et al and Costagliola et al mentioned above could be muscle contraction and inadequate fixation artifacts [41]. The authors were unconvinced that these 2 murine models constitute good GO model due to lack of macroscopic changes of proptosis and muscle hypertrophy. Due to small sample material, both orbital contents were subjected to RNA extraction and thus histology of this tissue compartment was not examined. Certainly in comparison with the control mice which were injected with empty plasmid, there was down regulation of IL5 expression in both mouse strains exposed to the TSHR and down regulation of IL4 and γIFN in TSHR immunized outbred mice. This would imply a relatively quiescent immunological environment within the orbital compartment. Nevertheless despite lack of rise in expression, TRAB measurements in terms of TSBAB and TBII titers were still correlated positively with the fold change in IL2 and γIFN in Balb/c mice and γIFN in outbred mice respectively.

2.2. Significance of findings in Balb/c and Swiss outbred mice

Swiss outbred mice provided a more heterogeneous genetic background, which made them less resistant to a TSAb response. A TSAb response in mice might be incompatible with the genetic background of the inbred Balb/c strain. There is evidence in humans that GD is a multigenic/multifactorial disease [50] and that genetic background is a major determinant of the ability to generate TSAbs. Moreover, a heterogeneous genetic makeup bears closer resemblance to human disease. Thus, Swiss outbred mouse is felt to be more suitable strain in generating such a GD or GO model. Balb/c model of GD may not be useful for the study of GO. However, it remains a useful tool for generation of antibodies recognizing the native structure of the human TSHR, with uses including the generation of mouse monoclonal TSHR antibodies and future TSHR studies [51].

2.3. Mouse GO versus Human GO

Bilateral orbital contents were utilized for RNA extraction leaving no room for histological examination of eye for verification of inflammatory changes. Neither could other imaging techniques such as computed tomography or magnetic resonance scanning be employed in this mouse study. Therefore it was not possible in this study to determine if histological or radiological characteristics of GO were present in the vaccinated mice. In addition, only histological resemblance between human GO and mouse GO were described in previous studies. No gross phenotypic description, in the lid or globe, was ever reported in mouse GO. Furthermore, mice and humans possess differences in the structures of their skeletal orbits which could contribute variations in response to eye inflammation. For example, physical characteristics such

as intra-ocular pressure, thought to play a role in pathogenesis of GO, is expected to vary in the 2 mammalian systems.

2.4. Clinical relevance of differences in cytokine profile in Mouse Model

Cytokines are produced in the thyroid and the orbit of patients with autoimmune thyroid diseases such as GD. This results in a continuation inflammatory reaction, which can directly affect thyroid function. In the outbred mice, the cytokine profile of thyroid was reported to be predominantly Th2 type with IL5 dominance [32]. These mice were TSAb positive, hyperthyroid, showed thyroid; all characteristic features of human GD. In the inbred mice [34], the cytokine profile present in the thyroid was Th1 with gamma interferon as the predominantly secreted cytokine. These mice produced mainly TSBAb and developed thyroiditis, a destructive inflammation of the thyroid gland. The findings in inbred mice mimicked human Hashimoto's thyroiditis. Parallels could be drawn from a study in human subjects by Heuer et al [52]. In humans, a Th1 response was demonstrated in Hashimoto's thyroiditis while a Th2 response was seen in thyroid of GD.

2.5. Limitation of current study

The mouse model of GO that was generated by Costagliola group [32] was adopted primarily because this remains the only reliable model in which histological features of GO could be reproduced associated with inflammatory infiltrates. However the minute amount of tissue samples from the orbit and thyroid proved the limiting factor in this study. We were only able to look into changes in cytokine expression by real time PCR method. Histology of thyroid and the eye were not done. Instead, TSHR antibody production was utilized as serological surrogate to

indicate presence of successful immunization. This problem could well be overcome by using bigger animals such as rats, rabbits or hamsters for experimentation. However, their immune response to a human TSHR may not be identical.

2.6. Possible Future Work / Experiments

Future work using macaques may offer additional benefits: 1) the structure of bony orbit in a macaque is similar to a human's, 2) the size of the sample makes available enough tissue for RNA collection as well as histology study, and 3) computed tomography and magnetic resonance imaging can potentially be done on these larger animals. However one potential shortfall in the primate model may be the close sequence homology between human and macaque TSHR, which could pose difficulty in inciting immunological response. Possible solutions include the use of methylated DNA for vaccination and usage of adjuvant. Once these problems are overcome, the primate model could be use to study factors affecting the pathogenesis of GO and testing out new immunomodulatory therapies which could revolutionize the treatment and prognosis in this disease.

VI. CONCLUSION

In conclusion, the verdict on the paradigm of Th1/Th2 response in GD and in particular, GO, is still out. Th1/Th2-type responses analyzed using two murine strains by the same method of immunization using the same plasmid prepared in identical ways, housed in the same animal holding area with no seasonal variation, fed with the same water and chow with the same iodine content provide conflicting observations. TRAB measurements by various modalities varied and the types of cytokine expression also differed. The only variance in this study is in the genetic makeup of outbred and inbred mice. It is possible that the genetic diversity in outbred mice contribute to a more plausible model for human GD.

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