INFLAMMATORY CYTOKINE REGULATION OF DEATH RECEPTOR-MEDIATED APOPTOSIS IN THYROID EPITHELIAL CELLS

Emese Mezősi, M.D.

Ph.D. Thesis

Supervisor: Endre V. Nagy, M.D., Ph.D.

Ist Department of Medicine

Medical and Health Science Center

University of Debrecen

2002

Table of Contents

ABBREVIATIONS

Ab, Antibody;

Ag, Antigen;

AITD, Autoimmune thyroid disease;

AMC, 4-amino-7-methylcoumarin;

BCA, Bicinchoninic acid;

BSA, Bovine serum albumin;

CFA, Complete Freund's adjuvant;

cFLIP, Cellular FLICE (FADD (Fas-associated via death domain)-like ICE (interleukin-1-beta converting enzyme))) inhibitory protein;

CHX, Cycloheximide;

CK, Cytokeratin;

DcR, Decoy receptor;

DISC, Death inducing signaling complex;

DR, Death receptor;

DTC, Differentiated thyroid cancer;

DTT, Dithio-threitol;

EAT, Experimental autoimmune thyroiditis;

ECL, Electrochemiluminescence;

EDTA, Ethylendiaminetetraacetic acid;

FADD, Fas-associated via death domain;

FasL, Fas ligand;

FBS, Fetal bovine serum;

FDA, Fluorescein diacetate;

FITC, Fluorescein isothiocyanate;

GAPDH, Glyceraldehyde-3 phosphate dehydrogenase;

H&E, Hematoxylin & eosine;

HT, Hashimoto's thyroiditis;

Hu-PBL-SCID, SCID mouse reconstituted by human peripheral blood lymphocytes;

IFNγ, Interferon γ;

Ig, Immunoglobulin;

IL-1β, Interleukin-1β;

IAP, Inhibitor of apoptosis protein;

NF-kB, Nuclear factor kB;

NK, Natural killer;

OVA, Ovalbumin;

PBMCs, Peripheral blood mononuclear cells;

PBS, Phosphate-buffered saline;

PI, Propidium iodide;

pTg, Porcine thyroglobulin;

SCID, Severe combined immunodeficient;

SDS, Sodium-dodecyl-sulphate;

SDS-PAGE, Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis;

TEC, Thyroid epithelial cell;

Tg, Thyroglobulin;

TIL, Tumor infiltrating lymphocytes;

TNFα, Tumor necrosis factor α;

TNF-R1, Tumor necrosis factor rececptor 1;

TRAIL, Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand;

TSH, Thyroid stimulating hormone;

1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. FasL- and TRAIL-mediated apoptosis

Apoptosis is a normal, active, genetically controlled process of cell death that does not require the participation of inflammatory processes (Arends 1991). Apoptosis mediates several normal functions in human biology including elimination of unneeded or unwanted cells in development, organ homeostasis, immune regulation and immune defense (Thompson 1995, Scaffidi 1999, Vaux 1999). Tissue homeostasis requires a proper balance between cell proliferation and cell death (Rudin 1997). Aberrant apoptosis is involved in the pathogenesis of many human diseases: abnormal cell death results in excessive parenchymal cell loss while decreased cell death contributes to the development of hyperplasias and neoplasias (Nagata 1996, Siegel 1999).

The two most frequently investigated apoptosis signaling pathways with relevance to thyroid homeostasis are the FasL (Fas Ligand) and the TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand) pathways (Arscott 1998, Mountz 1999, Bretz 2001). FasL and TRAIL are members of the tumor necrosis factor family and act through type I membrane proteins called death receptors (Itoh 1991, Wiley 1995). FasL is expressed in activated T lymphocytes and in cells of immune-privileged organs (Griffith 1995). It participates in cell-mediated cytotoxicity and maintenance of immune homeostasis by eliminating activated immune cells at the end of inflammatory reactions (Lowin 1994). A role for FasL-mediated apoptosis has been proposed for autoimmune thyroiditis (Giordano 1997, Hammond 1997, Borgerson 1999) and goiter involution (Tamura 1998). In contrast to FasL, TRAIL is expressed in a wide variety of normal tissues, suggesting that this pathway is highly regulated and protective mechanisms exist in normal cells (Griffith 1998a). While normal cells are resistant to TRAIL, it has been reported to selectively kill the majority of cancer cells (Gura 1997,

Ashkenazi 1999, Bonavida 1999, Walczak 1999). Recent studies suggest that the physiologic role of TRAIL is to remove virus-infected and cancer cells (Sedger 1999). TRAIL induces apoptosis by interacting with either of two death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) (Pan 1997, Walczak 1999). Two additional "decoy" receptors for TRAIL, that cannot transduce an apoptotic signal but can competitively block signal transduction, have also been identified: DcR1 (TRID, TRAIL-R3) and DcR2 (TRUNDD, TRAIL-R4) (Griffith 1998a).

During death receptor signaling the intracellular death domains of the receptors bind to an adapter protein that activates an apical caspase (Walczak 2000). This protein complex is known as death inducing signal complex (DISC). The initiator caspase cleaves and activates effector caspases that cleave other proteins, death substrates. This proteolytic cascade leads to apoptosis (Walczak 2000).

The death receptor pathways are regulated at several levels including the expression of death and decoy receptors, inhibitors of DISC formation and apical caspase activation as cFLIP (cellular FLICE Inhibitory Protein), caspase inhibitors as IAP (Inhibitor of Apoptosis Protein) protein family and bcl-2 family members (Bretz 2001).

Most recently, the important role of proteasome was established in the regulation of programmed cell death (Drexler 1997, Orlowski 1999). The proteasome is an ATP-dependent multisubunit proteolytic complex, responsible for the degradation of most intracellular proteins. Recent findings indicate that proteasome also plays a key role in cell cycle progression and in the activation of NF-kB which is an important modulator of cell survival during stress and immune responses (Palombella 1994). Proteasome inhibitors have been used to induce apoptosis in various cell types (Cui 1997, Van Valen 2000, Kim 2001), whereas in others, these compounds were able to prevent apoptosis induced by different stimuli (Lin 1998).

1.2. Death receptor-mediated apoptosis in autoimmune thyroiditis

The primary pathogenic event in the development of autoimmune endocrine diseases is the immune-mediated destruction of a particular type of endocrine cell (Foulis 1996, Mountz 1999, Ohsako 1999). Clinical disease in chronic autoimmune thyroiditis is caused by the specific cytotoxic destruction of thyroid epithelial cells (TECs) through apoptosis (Ohsako 1999, Borgerson 1999). This is a similar process to what appears to cause clinical illness in type I diabetes, where cytotoxic destruction of islet β cells leads to inadequate insulin production (Foulis 1996). Immune-mediated apoptosis, specifically triggered through the death receptor Fas, is believed to be central to the pathogenesis of autoimmune diabetes because apoptosis destroys islet β cells in both animal models of diabetes and the human type I disease (Yamada 1996, Benoist 1997, Giordano 1998). Animals that do not express normal Fas do not develop disease (Chervonsky 1997). Recent evidence suggests that this is due to loss of target cell susceptibility to apoptosis rather than disruption of the immune response by alteration of Fas (Su 2000).

Fas-mediated destruction has also been proposed as the cause of hypothyroidism in chronic autoimmune thyroiditis (French 1997, Giordano 1997, Bretz 1999a). Other death receptors, such as DR4 and DR5 may also be involved in this process, but due to their very recent characterization, have not been evaluated. Activated T lymphocytes expressing death ligands such as FasL and TRAIL may be responsible for inducing thyroid cell destruction during the inflammatory response to thyroid autoantigens. Thyroid cells from patients with autoimmune thyroiditis display an increased frequency of apoptotic cells (Dremier 1994, Okayasu 1995, Tanimoto 1995, Kotani 1995). Many of the apoptotic cells in these glands are found in the areas of disrupted follicles, in proximity to infiltrating lymphoid cells (Kotani 1995, Hammond 1997). This suggests

that the increase in thyroid follicular cell apoptosis in thyroiditis may occur through a mechanism related to immune responses and inflammation, and may underlie thyroid destruction.

1.3. The role of inflammatory cytokines in autoimmune thyroiditis

It has been shown that inflammatory cytokines are involved in the regulation of apoptosis (May 1997). Cytokines can influence the expression of apoptotic signaling components and inhibitors in target cells, as well as control the expression of apoptotic initiators in effector cells (Bretz 2001). Many inflammatory cytokines are present in the thyroid gland in autoimmune thyroid disease, and there is evidence that these cytokines play an important role in the development of this process (Kawakami 1990, Frohman 1991, Ajjan 1996, Alimi 1998). It has been demonstrated that T cell clones isolated from intrathyroidal lymphocytic infiltrates of Hashimoto's thyroiditis (HT) produce high levels of IFNγ and TNFα (Del Prete 1989, Roura-Mir 1997), and cytokine-producing lymphocytes also are observed adjacent to thyrocytes (Hamilton 1991). Furthermore, IFNγ in combination with TNFα impairs the growth of thyrocytes (Weetman 1988). Several groups have published results on the influence of inflammatory cytokines on Fas-mediated apoptosis of cultured thyroid cells (Kawakami 1996, Giordano 1997, Bretz 1999a, Paolieri 1999). It was also shown that thyroid epithelial cells are susceptible to TRAIL-mediated apoptosis after pretreatment with cycloheximide (CHX) and that TRAIL itself is expressed by the TECs treated with IFNγ, TNFα or IL-1β (Bretz 1999b). Regulation of death receptor pathways in the thyroid may be a potential mechanism in which inflammatory cytokines might act to promote disease progression.

1.4. Murine model of experimental autoimmune thyroiditis

Thyroiditis can be experimentally induced in mice bearing the H-2K haplotype by immunization with thyroglobulin (Tg) and adjuvants (Vladutiu 1971). In this experimental autoimmune thyroiditis (EAT) model, mice develop autoimmune responses characterized by the occurrence of circulating anti-Tg antibodies and infiltration of the thyroid gland by lymphoid cells, including $CD4^+$ and $CD8^+$ T cells. EAT mimics some of the immunologic manifestations of Hashimoto's thyroiditis but at variance with the human disease it regresses spontaneously after several weeks without thyroid follicular disruption, and is not accompanied by signs of hypothyroidism (Weigle 1980, Charreire 1989).

Inflammatory cytokines are present in inflamed thyroids and have been shown to influence thyroid disease in experimental autoimmune thyroiditis animal models (Ajjan 1996). Several publications have suggested a crucial role for cytokines in the pathogenesis of EAT (Tang 1993, Kawakami 1990, Frohman 1991, Alimi 1998, Stull 1992, Schuppert 1996) but the exact function of these molecules is not clear. There has also been no information on the effect of the administration of both IFNγ and TNFα on the thyroid *in vivo*.

1.5. Apoptosis in the pathogenesis of goiter nodules

Nodular goiter is one of the most common endocrine diseases. The pathogenesis of nodule formation has been intensively studied and recently activating mutations in the TSH receptor and Gsα genes were identified in the development of toxic adenomas (Derwahl 1996a). However, the majority of non-hyperthyroid nodules do not demonstrate protooncogene mutations and the primary events in the pathogenesis of these nodular goiters are still unknown (Derwahl 1996b). The most widely accepted

hypothesis of nodule formation argues that there is heterogeneity in the growth potential and function of individual thyrocytes (Studer 1995). Increasing evidence suggests a role for growth factor production in the thyroid, leading to TSH-independent growth of thyroid nodules (Maiorano 1994, Gartner 1996, Bidey 1999). A lack of growth inhibition may also participate in the tissue imbalance that results in nodular goiter (Asmis 1996).

Normal thyroids show a low level of apoptosis, a possible result of basal thyroid cell turnover (Tanimoto 1995, Dremier 1994). In multinodular goiters and thyroid adenomas similarly low apoptosis rates have been detected by immunohistochemistry (Kikuchi 1997, Moore 1998, Sreelekha 2000). Normal primary thyrocytes are resistant to both TRAIL and FasL-induced apoptosis, despite the constitutive expression of their respective death receptors (Arscott 1997, Bretz 1999b). However, thyroid cells can be sensitized by IFNγ/IL-1β or TNFα/IL-1β to undergo apoptosis in response to FasL or TRAIL, respectively (Bretz 1999a, Bretz and Mezosi 2002). No studies have investigated the regulation of death receptor-mediated apoptosis in nodular goiter.

1.6. SCID mouse model for *in vivo* studies of human thyroid cancers

The limitations of animal models in cancer research are well known. Virus or chemically induced, highly antigenic experimental tumors in animals are irrelevant to spontaneously developing human tumors which are frequently nonimmunogenic (Kripke 1988). While *in vitro* systems provide a wealth of information about cellular and molecular biology of tumor cells, they are inadequate to study the complexity of human neoplastic diseases, the metastatic features, the experimental therapeutics and anti-tumor immunity (Williams 1993). The immunodeficient mice provided unique possibility to investigate transplanted human cancers *in vivo* (Mueller 1991,

Hendrickson 1993). The first attempt was the use of athymic nude mice, but the success rate of transplantation was variable, the model was not suitable for all types of tumors and metastases usually did not develop (Mueller 1991, Hendrickson 1993, Volpe 1993). While nude mice accept human solid tissues, all human lymphocytes are killed because of the presence of functional murine natural killer (NK) cells and B lymphocytes (Volpe 1993). Severe combined immunodeficient (SCID) mice are carrying an inherited defect of the recombinase system for antigen receptor genes (Bosma 1983). The receptor defect results in a complete lack of mature T and B cells in these mice. Therefore, SCID mice accept both human solid tissues and lymphocytes. The multitude of human cancers (lymphoma, leukaemia, lung, breast, ovarian, colon cancer, retinoblastoma, osteosarcoma, melanoma) was successfully transplanted in SCID mice (Mueller 1991, Hendrickson 1993, Juhasz 1993, Volpe 1993, Paine-Murrieta 1997).

The implantation of human immune cells results in the repopulation of the mouse bone marrow and lymphoid organs by human lymphocytes (Martin 1992, Hendrickson 1993, Mosier 1988). The process is called immune 'reconstitution', referring to the largely uncharacterized presence of the human immune system in these mice. There are several methods to accomplish immune reconstitution. In the present experiment, human peripheral mononuclear cells were injected intraperitoneally (hu-PBL-SCID mice). Human B cells are able to produce human immunoglobulins several months after the reconstitution (Martin 1992, Mosier 1988).

The immune system plays a crucial role in the control of malignant diseases. The hu-PBL-SCID mouse model was found to be suitable for the investigation of T-cell specific and natural anti-tumor immune responses (Iwanuma 1997, Tam 1999). Recently, promising results of immuno- and immunocytokine therapy of leukemia, lymphoma,

melanoma, ovarian and breast cancer were published using this animal model (Bauer 1999, Bonnet 1999, Cochlovius 1999, Hu 1999, Schultes 1999).

Differentiated thyroid cancer (DTC) is of special interest in tumor immunology (Juhasz 1989, Baker 1993, Baker 1995, Boyd 1996). DTC is present in situ in 10% of all autopsy specimens, indicating that the preclinical form of the disease is very common (Bisi 1989). Lymphocytic infiltration is frequently observed in and around the tumor. The lymphocytic infiltration is believed to be an immunologic reaction induced by antigens from the carcinoma itself (Aoki 1979, Kamma 1988). In thyroid cancer patients with thyroiditis a better survival rate is observed (Kashima 1998). HLA-DR antigens are expressed on tumor cells and on peritumoral lymphocytes (Goldsmith 1988, Matsubayashi 1995, Feinmesser 1996). The association of DTC with certain HLA-DR alleles was reported (Juhasz 1989). On the surface of tumor cells thyroid specific antigens can be found: thyroglobulin, thyroid peroxidase, TSH receptor (Baker 1995). These thyroid specific antigens have been well documented to be the targets of autoimmune thyroid diseases (Baker 1993). Thyroid autoantibodies may occur in thyroid cancer patients (Kamma 1988, Baker 1995). Interestingly, papillary cancer was one of the first tumors where immunotherapy was attempted (Amino 1975).

Regulation of tumor cell apoptosis is a promising way to improve anti-cancer therapy. The presence of death ligands on tumor infiltrating lymphocytes and the contribution of death receptor-mediated apoptosis to tumor cell killing in DTC is unknown. TRAIL was found effectively kill carcinoma cell lines originating from the follicular thyroid epithelium (Mitsiades 2000). However, as a reliable animal model of this disease was not available, no studies on primary thyroid tumors have been done regarding the induction of apoptosis or other forms of immunotherapy.

2. SPECIFIC AIMS

In the present studies we sought to examine

1. the inflammatory cytokine regulation of TRAIL-mediated apoptosis in human thyroid epithelial cells
2. the mechanism of IL-1β/TNFα-induced sensitization of thyroid cells to TRAIL
3. the expression of TRAIL and its receptors in the normal thyroid gland and in thyroid tissue from Hashimoto's thyroiditis
4. the effect of IFNγ/TNFα on the Fas-mediated apoptosis and thyroid destruction in a murine model of experimental autoimmune thyroiditis
5. the sensitivity of primary thyroid cells from goiter nodules to TRAIL- and Fas-mediated apoptosis
6. the influence of proinflammatory cytokines on goiter cell apoptosis
7. the regulation of TRAIL pathway in goiter cells
8. the proteasome activity in primary thyroid cells derived from normal thyroids, multinodular goiters and papillary cancers
9. the usefulness of the hu-PBL-SCID mouse model for the investigation of human thyroid tumors and interactions between the immune system and the tumor cells.

3. MATERIALS AND METHODS

3.1. Cell Culture Experiments

3.1.1. Cell culture

The studies and tissue procurement were approved by the University of Michigan Institutional Review Board. Normal thyroid tissue was obtained from patients at thyroidectomy from the uninvolved, contralateral lobes of thyroids resected for tumors (n=10, age: 42.3+14.3 y, F/M: 9/1). Goiter cells were derived from well-defined nodules of multinodular goiters (n=17, age: 51.5+13.4 y, F/M: 17/0). The histological diagnosis was nodular hyperplasia in every case. The non-nodular part of multinodular goiters was also obtained and the primary cultures from these tissues were usable in ten cases. Three papillary cancers were used for screening of proteasome activity in tumor tissue (age 45.1+9.7 y, F/M: 3/0). Thyroid tissue was digested overnight with 40 mg collagenase, 4 mg hyaluronidase, and 4 mg DNase I (all from Sigma Chemical Co) in 40 ml RPMI-1640 (GIBCO-BRL). Red blood cells were lysed with ammonium chloride lysis buffer (0.15 M NH_4Cl, 10 mM KPO_4, and 1 mM EDTA, pH 7.3), and cells were cultured in CellGro Complete media (Mediatech) supplemented with 20% NuSerum IV (Collaborative Biomedical Products), 100 units/ml penicillin, 100 μg/ml streptomycin and 10 mIU/ml bovine TSH (Sigma) at 37 ^oC in 5% CO_2. After 24 h, nonadherent cells were removed by washing with CellGro Complete media. Nuserum IV is a partly artificial serum which contains 25% fetal calf serum, so the final concentration of fetal calf serum in the culture medium was 5%. The purity of thyroid cell population was checked by anti-cytokeratin 18 (CK18) antibody (a marker for epithelial cells), quantitated by flow cytometry and only cultures that were >90% cytokeratin positive were used for experiments.

3.1.2. Cytokines, TRAIL, agonist antibodies, soluble receptors and proteasome inhibitors

Primary thyroid cells were treated for four days with cytokines at the following concentrations: 100 U/ml IFNγ (Roche Molecular Biochemicals), 50 ng/ml TNFα (Collaborative Biomedical Products), 50 U/ml IL-1β (Sigma Chemical Co.). Cells were then treated overnight with 800 ng/ml TRAIL, 0.1-0.5 μg/ml agonist goat polyclonal anti-DR5 antibody (R & D Systems) or 1 μg/ml agonist mouse monoclonal IgM anti-Fas antibody (clone CH11, Upstate Biotechnology). TRAIL (a kind gift of A. Chinnaiyan) was affinity purified as described from bacterial lysates of cells transformed with the plasmid pET15b-His-FLAG-TRAIL. The purity of the TRAIL preparation was confirmed by silver stained SDS-PAGE (sodium-dodecyl-sulphate polyacrylamide gel electrophoresis) and limulus amoebocyte lysate assay (Chinnaiyan 2000). Soluble human DR5/IgG Fc chimera, used as specific inhibitor of TRAIL, and human TNF-R1/IgG Fc chimera were kindly provided by R&D Systems. In order to detect the effect of proteasome inhibition on TRAIL susceptibility, two different proteasome inhibitors, lactacystin and MG132 (Calbiochem) were used at 1 μM and 10 μM concentrations, respectively, at the same time of TRAIL administration.

3.1.3. Determination of cell viability and apoptosis

Cell death was measured 20 h after TRAIL, anti-DR5 or anti-Fas antibody administration except for otherwise indicated. In studies with proteasome inhibitors, the measurement was 5 h after TRAIL treatment, to avoid the toxicity of proteasome inhibition. Cell viability was determined by staining with fluorescein diacetate (FDA) and propidium iodide (PI), and quantitated by flow cytometry as described by Killinger (Killinger 1992). Living cells are FDA positive and PI negative. During the apoptosis

process, cells lose the capability of FDA uptake while maintaining membrane integrity and become FDA negative and PI negative. In the late phase of apoptosis, parallel with the increase of membrane permeability, cells will be FDA negative and PI positive. Flow cytometric analysis of caspase-specific proteolytic cleavage of cytokeratin 18 (M30 CytoDEATH® monoclonal Ab) and Annexin V binding (Annexin V-FLUOS staining kit) were used as described by the manufacturer to confirm that apoptosis had occurred (both from Roche Molecular Biochemicals).

3.1.4. RNase protection assay

RNA was isolated from cells using Trizol Reagent according to the manufacturer's protocol (GibcoBRL). RiboQuant MultiProbe RNase Protection Assay System (BD PharMingen) was used for the detection and quantitation of TRAIL receptors (hAPO-3d template set) and IAPs (hAPO-5 template set). ^{32}P-labeled antisense RNA probes were prepared and hybridized with 2-5 μg total RNA from primary cultures of thyrocytes. After hybridization, the samples were subjected to RNase treatment followed by purification of RNase-protected probes. The protected probes were resolved on a 5% denaturing polyacrylamide gel. We quantified transcripts by autoradiography followed by densitometry (Quantity One, Bio-Rad Laboratories). The relative signal intensity was corrected for RNA loading by comparison with the GAPDH band intensity for each sample.

3.1.5. Immunoblot analysis

RIPA lysis buffer (1% NP40, 0.5% Na-deoxycholate, 0.1% SDS in PBS) was used for the detection of TRAIL receptors and cFLIP, Triton-X lysis buffer (150 mM NaCl, 10 mM Tris (pH=7.4), 5 mM EDTA, 1% Triton-X-100) to determine bcl-2 protein level,

with protease inhibitors (Complete, Roche Molecular Biochemicals). Insoluble material was removed by centrifugation and supernatants were stored frozen at –20 ^{o}C until used for Western analysis. Total protein concentration was quantitated by BCA (bicinchoninic acid) protein assay kit (Pierce Chemical Co.) and equivalent amounts of each sample were electrophoretically separated on a 12.5% polyacrylamide gel and transferred to nitrocellulose membrane. Goat polyclonal anti-DR5 (R&D Systems), mouse monoclonal anti-DR4 (Imgenex), rabbit polyclonal anti-DcR1 (Affinity BioReagents), rabbit polyclonal anti-DcR2 (ProSci), rabbit polyclonal anti-cFLIP (Alexis) and hamster monoclonal anti-bcl-2 antibodies (BD PharMingen) were used according to the manufacturers' protocol. The results were visualized by ECL (electrochemiluminescence) reaction (Amersham) followed by autoradiography.

3.1.6. Flow cytometric determination of cytokeratin 18

Total cytokeratin 18 expression was determined as described by the vendor (Chemicon) of the antibody. Briefly, trypsinized cells were washed and fixed in ice-cold methanol for 30 min, washed in PBS and incubated for 15 min in blocking buffer (2% FBS in PBS). Blocking buffer was replaced with anti-CK 18 antibody diluted to 2.0 μg/ml in blocking buffer and incubated for 1h. Cells were then washed in blocking buffer and resuspended in anti-mouse FITC conjugate (Jackson ImmunoResearch) diluted to 1:100 in blocking buffer for 30 min. A mouse IgG1 (MOPC21, Sigma) was used as an isotype-matched control antibody.

3.1.7. Flow cytometric determination of DR5, DR4, DcR2 and DcR1 surface expression

Cytokine treated thyroid cells were made non adherent by incubation with 0.265 mM EDTA in PBS, washed in PBS and incubated for 15 min in blocking solution (2% normal horse serum, 1% BSA in PBS) at 4 ^{o}C. Goat polyclonal anti-human DR5, DR4, DcR2 or DcR1 antibodies (R & D Systems) were diluted in blocking solution to 5 μg/ml and cells were incubated with the antibodies for 20 min, at 4 ^{o}C. After washing in the blocking solution, FITC-conjugated anti-goat F(ab')2 fragment (Jackson ImmunoResearch) was used in 1:100 dilution for 20 min, at 4 ^{o}C. Purified goat IgG (R & D Systems) served as control. $2x10^4$ cells were acquired for each sample and quantitated on a FACScalibur flow cytometer (Becton Dickinson).

3.1.8. Immunostaining of thyroid sections

The expression of TRAIL and its receptors on human thyroid tissue sections was analyzed by immunohistochemical staining. Formalin-fixed, paraffin-embedded sections were obtained after pathological examination from normal thyroid glands and from patients with chronic autoimmune thyroiditis. Sections were deparaffinized with three rinses of xylene and rehydrated in ethanol. For antigen unmasking the slides were microwave-treated for 15 min in 0.01 M citrate buffer pH=6.0. Neutralization of endogenous peroxidases was performed by 30 min incubation of sections with 3% H_2O_2 dissolved in methanol. Samples were blocked with 5% goat or horse serum (depending on the secondary antibody) for 20 min. DR5 expression was investigated by two rabbit polyclonal antibodies against different epitopes of DR5 (YProSci Inc., recognizing amino acids 388 to 407 of human DR5 and R&D Systems, recognizing amino acids 139-152 of human DR5). DR4, DcR2 and DcR1 were detected by rabbit polyclonal

antibodies from BD PharMingen, YProSci Inc. and Affinity BioReagents, respectively. Rabbit IgG antibody was used as a control (Jackson ImmunoResearch Laboratories). The slides were incubated with rabbit antibodies for 1 h at 5 μg/ml in 1.5% normal goat serum. After washing with PBS, the slides were incubated with biotinylated goat anti-rabbit IgG and detected using avidin-biotin complex kit with VIP substrate (Vector Laboratories). TRAIL expression was examined using mouse monoclonal antibody (BD PharMingen) and MOPC 141 antibody as an isotype (IgG2b) matched control (Sigma), at 5 μg/ml in 1.5% normal horse serum for 1h. Secondary antibody was biotinylated horse anti-mouse IgG (Vector Laboratories). Slides were counterstained with methyl green and mounted with Permount (Sigma).

3.1.9. Immunostaining of cultured thyroid cells

Thyroid cells were grown on Falcon chamber slides (Becton Dickinson). For immunostaining, slides were washed twice with PBS then fixed with methanol for 5 min at 4 ^{o}C, briefly air dried and blocked with 5% normal goat serum in PBS. Slides were incubated with 1.0 μg/ml affinity-purified rabbit anti DR5 antibody (YProSci Inc.) or ChromPure rabbit IgG (Jackson ImmunoResearch Laboratories) in 1.5% normal goat serum in PBS for 18 h at 4 ^{o}C. After washing with PBS, the slides were incubated with biotinylated goat anti-rabbit IgG and detected using avidin-biotin complex kit with VIP substrate (Vector Laboratories). Slides were counterstained with methylgreen and mounted with Permount (Fisher Scientific).

Laser scanning confocal microscopy of cultured thyroid cells was performed at the University of Michigan Microscopy and Image Analysis Laboratory on a Zeiss model LSM 510 microscope. For confocal microscopy, thyroid cells cultured in chamber slides were fixed in ice-cold methanol for 10 min. Samples were blocked in 10% FBS

for 20 min and the following antibodies were used for the detection of TRAIL receptors: anti-DR5 rabbit polyclonal antibody (R & D Systems) and anti-DcR1 rabbit polyclonal antibody (ABR), at 5 μg/ml concentration. The slides were incubated with the primary antibodies overnight at 4 ^{o}C. After washing in PBS the cultures were incubated with Texas-Red-X conjugated anti-rabbit secondary antibody (Molecular Probes) at 10 μg/ml for 30 min. The samples washed in PBS and mounted in Prolong Antifade mounting media (Molecular Probes) were stored at 4 ^{o}C until analysis.

3.1.10. Fluorogenic peptide substrate assay for proteasome activity

Thyroid cells were lysed and homogenized in ice-cold buffer A (50 mM Tris-HCl, pH=7.5, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA). Protein concentration was quantitated by BCA protein assay kit (Pierce Chemical Co.). Proteasome substrate Suc-LLVY-AMC (Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin) (Calbiochem) was incubated at a final concentration of 0.1 mM with thyroid cell lysates containing 5 μg protein at 37 ^{o}C for 30 min. The reaction was stopped by buffer F (30 mM Na-acetate, pH 4.3, 100 mM $CH_2ClCOOH$, 70 mM acetic acid). Hydrolysis of peptides was determined with a RF-5001 PC spectrofluorometer (Shimadzu, Tokyo, Japan) at 380 nm excitation/460 nm emission for AMC. The measurements were made in triplicate. Results were standardized to hydrolysis of Suc-LLVY-AMC by 5 μg recombinant proteasome (Calbiochem) which was used as a positive control (100%).

3.2. Animal Studies

3.2.1. Induction of EAT

Eight-week-old female CBA/J mice, a strain susceptible to EAT, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions with free access to food and water. They were allowed to adapt to their environment for 1 week before initiating the experiment. Mice were s.c. challenged at the base of the tail with 100 μg of porcine Tg (pTg; Sigma-Aldrich) emulsified in complete Freund's adjuvant (CFA) (Difco). Two weeks later, the mice were boosted with the same dose of pTg in incomplete Freund's adjuvant. Control animals were injected with ovalbumin (OVA) (Sigma-Aldrich) in adjuvant at the same time intervals as pTg-injected animals. One week after the booster immunization, mice were i.p. injected with 5 μg of mouse recombinant IFNγ (R&D Systems), 0.5 μg of mouse recombinant TNFα (R&D Systems), or 5 μg of IFNγ and 0.5 μg of TNFα in PBS for 3 consecutive days. Control animals were injected with BSA in PBS.

3.2.2. Serum anti-Tg antibody determination

Anti-Tg antibodies were assayed by solid-phase ELISA. Serial dilutions of sera collected before immunization, 2 weeks after initial immunization, and before sacrifice were used to determine endpoint titers. Briefly, 96-well microtiter plates were coated with pTg, and the plates were then blocked by adding BSA. After washing the wells, sera from individual mice, diluted 1/200 000, were added and incubated overnight at 4°C. Alkaline phosphatase-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories) was added as second antibody and the colorimetric reaction was revealed by the addition of p-nitrophenyl phosphate substrate (Sigma-Aldrich). The plate was quantitated with an ELISA reader at 405 nm. Murine Tg was

isolated from CBA/J mice by M-per mammalian protein extraction reagent (Pierce), then run on SDS-polyacrylamide gel, and immunoblot analysis was performed with mouse serum (diluted 1/4000) prepared from pTg-immunized mice treated with or without cytokines. A commercial rabbit anti-Tg (DAKO) was used as a positive control.

3.2.3. Thyroid histopathology

Thyroid glands were fixed in 10% formalin, embedded in paraffin, or directly frozen in Tissue-Tek OCT (Sakura Finetechnical) and sectioned by standard method. Infiltration was evaluated on 5-μm-thick sections stained with hematoxylin and eosine (H&E). The severity of thyroiditis was graded on a scale of 0–4, as follows (Batteux 1999): grade 0, normal histology; grade 1, interstitial accumulation of inflammatory cells distributed around one or two follicles; grade 2, one or more foci of inflammatory cells reaching at least the size of one follicle; grade 3, 10–40% of thyroid replaced by inflammatory cells; and grade 4, >40% of the thyroid replaced by inflammatory cells. Scoring was performed blind to the animal treatment groups.

3.2.4. TUNEL staining and immunohistochemistry

Apoptosis in thyroid sections was detected by TUNEL staining of fragmented DNA. Specific staining for in situ apoptosis was performed using the ApopTag peroxidase kit (Intergen), according to the manufacturer's protocol. The infiltrating immune cells in mouse thyroid were evaluated for CD45, CD4, and CD8 expression by immunohistochemical staining. The paraffin-embedded sections were used to stain for CD45, whereas the frozen sections were used to stain for CD4 and CD8. Briefly, after the endogenous peroxidase was neutralized by 3% H_2O_2, slides were blocked for

nonspecific binding by histomouse blocking solution (Zymed Laboratories) and incubated with biotin-conjugated rat monoclonal anti-mouse CD45 (Ly-5), CD4 (L3T4), or CD8 (Ly-2) antibodies or isotype-matched rat IgG2b or IgG2a (BD PharMingen). The sections were incubated with streptavidin-peroxidase conjugate and were developed by incubation with diaminobenzidine substrate. The slides were counterstained with hematoxylin and mounted with GVA MOUNT (Zymed Laboratories).

3.2.5. Thyroid injection with anti-Fas antibody

For direct injection of antibody into the thyroid, mice were anesthetized with ketamin (0.5 mg/g) and xylazine (0.05 mg/g) i.p. Mice were pretreated with 5 μg of IFNγ and 0.5 μg of rTNFα or BSA in PBS for 3 consecutive days before anti-Fas antibody application. Using sterile procedures, a lateral neck dissection was performed to visualize the thyroid gland and dissect it free of surrounding fascia, connective tissue, or muscle while maintaining its intact capsule and blood supply. Either anti-murine Fas (Jo2) or control IgG (BD PharMingen) was injected directly into the thyroid gland using a Hamilton 50-μl syringe. The wound was closed in layers using conventional surgical procedures and, after 8 h, mice were sacrificed and thyroid tissues were harvested for analysis of apoptosis by TUNEL staining.

3.2.6. Engraftment of human thyroid tumors into SCID mice

Tumor tissue samples were obtained from ten patients with suspected thyroid malignancy during operation. Data of patients, histological types and cancer stages are shown in Table 1. The experimental protocol was approved by the University of Debrecen Institutional Review Board. All patients gave a written informed consent.

C.B-17-scid/scid mice were bred at our animal care facility and housed in microisolator cages. All mice were maintained on sterilized food and water without any antibiotics.

Table 1. Patients' data

No	Age	Sex	Histology	Stage
1.	50	F	Papillary	T1aN1aM0
2.	32	F	Papillary	T2aN0M0
3.	23	F	Papillary	T1bN1aM0
4.	59	M	Papillary	T3aN0M0
5.	30	M	Papillary	T3aN1aM0
6.	29	F	Follicular	T2aN0M0
7.	42	M	Medullary	T2bN1bM0
8.	64	F	Medullary	T1aN1aM0
9.	82	F	Anaplastic	T4N0M0
10.	19	F	Adenoma	

At the age of 4-6 weeks blood samples were taken from the retrobulbar vessels for the determination of murine immunoglobulin levels. Leaky mice (IgM $\geq$ 1 μg/ml) were excluded from further investigations. Mice were 8-12 weeks old at the time of xenograftment. One 8x4x3 mm sample from each thyroid tumor was cut into two pieces of identical size and transplanted into two SCID mice. A subcutaneous pocket was prepared on the lateral abdominal wall under methoxyflourane anesthesia. The tumor tissue was carefully placed in the pocket. The skin was sutured with non-absorbable suture and covered with bandage until wound healing. The presence and size of the engrafted tumor tissues were checked by palpation and recorded regularly. After 16 weeks, the animals were sacrificed by exsanguination under anesthesia. Organs of the animals were checked for the presence of local and distant metastases. The size and weight of each removed tumor implant were recorded.

3.2.7. Reconstitution of the human immune system

In the case of each tumor, one of the two mice (mouse A) has been injected intraperitoneally with peripheral blood mononuclear cells (PBMCs), $2x10^7$ cells/animal,

separated from the blood of the same tumor patient on day 1 postoperatively by Ficoll-Hypaque density gradient centrifugation. The other animal with the same tumor (mouse B) has not received human lymphocytes.

3.2.8. Murine and human immunoglobulin measurements

Murine IgM was measured by ELISA. Rat anti-mouse IgM heavy chain (Serotec) was used as coating antibody and peroxidase conjugated rabbit anti-mouse IgM (Serotec) as secondary antibody.

For human IgG ELISA, mouse anti-human IgG heavy chain monoclonal antibody (Serotec) was coated on 96 well ELISA plates. A peroxidase conjugated goat anti-human IgG Fc specific antibody (Serotec) was added as secondary antibody and the colorimetric reaction was revealed by the addition of orthophenylendiamine substrate.

3.2.10. Histology, immunohistochemistry in the SCID mouse model

The original human tumors (before transplantation), the explanted thyroid tissues and the organs of mice were fixed in 4% formalin and paraffin-embedded sections were investigated using histology and, in the cases of DTC immunohistochemistry. Immunohistochemistry was performed using the immunoperoxidase technique (VECTASTAIN Universal Elite kit peroxidase, VECTOR VIP kit substrate). Monoclonal antibodies to human markers included anti-CD3 (Novocastra), anti-CD8, anti-CD20, anti-CD45RO, anti-CD68, anti-HLA-DR and anti-thyroglobulin from DAKO.

3.2.11. Computer software and statistical analysis

Flow cytometry data were analyzed by WinMDI 2.8 (Joseph Trotter URL http://facs.scripps.edu/). Densitometric quantitation of autoradiograms was performed using Quantity One (Bio-Rad). Statistical analysis was performed using χ^2-test, Student's t-test and Wilcoxon matched pairs test using Stat View software (Abacus Concepts, Berkeley, CA). A p value of <0.05 was taken as statistically significant.

4. RESULTS

4.1. TNFα combined with IL-1β induces susceptibility to TRAIL-mediated apoptosis in thyroid cells

It has been demonstrated previously that both FasL and TRAIL are capable of inducing apoptosis in TECs after CHX treatment (Arscott 1997, Bretz 1999b). It was also shown that IFNγ combined with TNFα and/or IL-1β pretreatment of TECs could provide death susceptibility to Fas-mediated apoptosis (Bretz 1999a). In order to determine whether TRAIL-mediated apoptosis could be similarly induced by inflammatory cytokines we treated normal (obtained from non diseased tissue) primary TEC cultures for four days with various combinations of cytokines prior to addition of recombinant TRAIL to the culture medium. Substantial cell death occurred in cultures pretreated with the combination of TNFα and IL-1β, with lower but significant levels of cell death detectable in IL-1β treated cultures (Fig. 1).

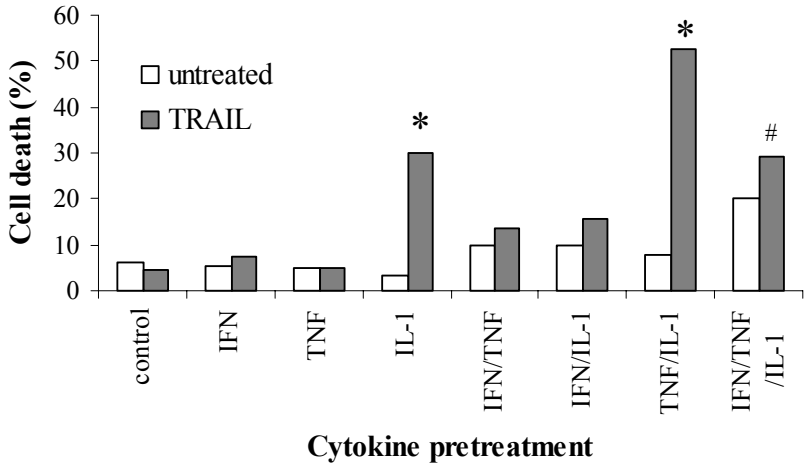


Figure 1. *Inflammatory cytokines induce susceptibility to TRAIL-mediated cell death in TECs* Normal primary TEC cultures were pretreated for 4d with the indicated cytokines prior to addition of TRAIL (800 ng/ml). Cells were stained with PI and FDA for quantitation by flow cytometry. 10 000 cells/sample were assayed for live and dead cells. Asterisks denote results of TRAIL treatment with $p < 0.0001$ when compared separately with both the control sample (no cytokines) and untreated (cytokines but no TRAIL). The # symbol denotes the result of TRAIL treatment with $p < 0.0001$ when compared with TNF/IL-1/TRAIL treatment. This assay is

representative of results from ten independent experiments each using thyroid cultures from different patient samples.

No cell death was observed with TNFα pretreatment. Optimal concentrations of IL-1β and TNFα were used suggesting the combination of the two cytokines results in a true synergistic effect rather than an additive effect. The additional pretreatment of these cultures with IFNγ inhibited cell death in both circumstances (Fig. 1). The percentage of cell death occurring under these conditions varied in cultures derived from one patient sample to another but in all ten samples tested the requirement for these particular cytokine combinations and only these combinations was absolute. Fig. 2 shows the time-course of inflammatory cytokine pretreatment for induction of thyroid cell susceptibility to TRAIL. TRAIL-mediated cell death occurred after three days of cytokine pretreatment with maximum detectable death occurring after four days pretreatment. The requirement for at least three days exposure of the TEC cultures to TNFα/IL-1β combined with the rapid (<4h, Fig. 7 and 8) cell death after treatment with TRAIL suggests that TRAIL is the primary death signal rather than TNFα.

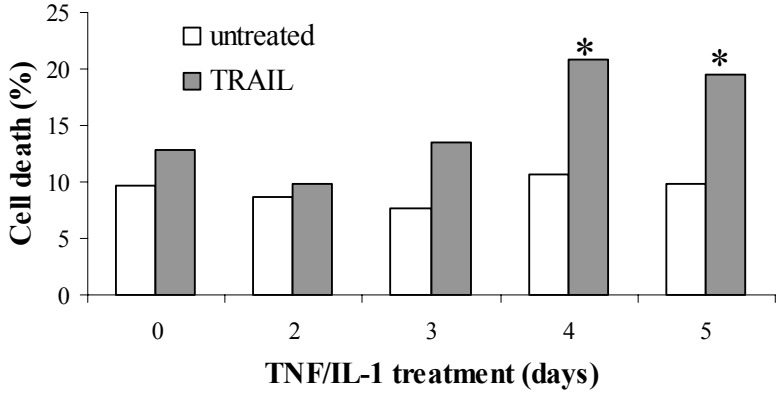


Figure 2. *Time course of TNFα/IL-1β induced susceptibility to TRAIL-mediated cell death*
Cell cultures were treated with TNFα/IL-1β for the indicated number of days prior to the addition of TRAIL and cells were harvested after 6h incubation with TRAIL. Asterisks denote results of TRAIL treatment with $p < 0.0001$ when compared separately with both the control sample (day 0) and untreated (cytokines but without TRAIL).

To further distinguish whether the primary death signal is from TRAIL and not TNFα we reversed the treatment conditions by exposing the cells to TRAIL and IL-1β for four days prior to TNFα treatment and compared cell death to the previously defined conditions. Data in Fig. 3 shows that pretreatment with TRAIL and IL-1β does not impart susceptibility to TNFα-mediated cell death.

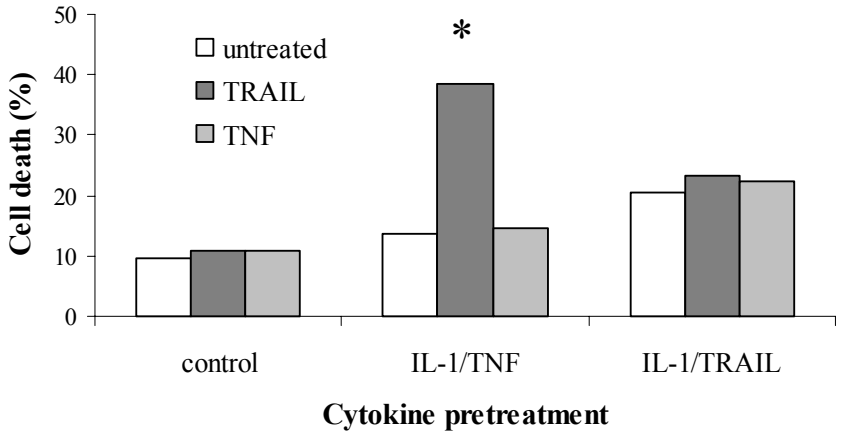


Figure 3. *TRAIL and not TNFα provides the death signal*
Cells were treated for 4d with TNFα/IL-1β or TRAIL/IL-1β and assayed as in Fig.1 prior to the addition of TRAIL (800 ng/ml) or TNFα (50 ng/ml). The asterisk denotes results of TRAIL treatment with $p < 0.0001$ when compared separately with all other samples.

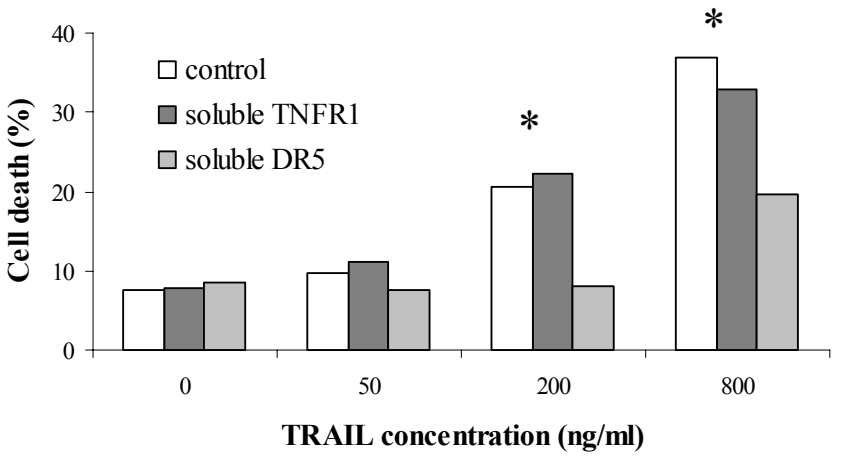


Figure 4. *Specificity of TRAIL-mediated apoptosis*
Cells were treated for 4d with TNFα/IL-1β and were preincubated with 0.2 μg/ml TRAIL-neutralizing soluble DR5 or soluble TNFR1 for 1h prior to addition of TRAIL to cultures, at concentrations displayed in the figure. Cell death was measured as in Fig. 1. The asterisks denote results of TRAIL + soluble TNFR1 treatment with $p < 0.0001$ when compared separately with untreated (no TRAIL) control and cultures with the same concentration of TRAIL with soluble DR5.

To demonstrate the specificity of the recombinant TRAIL preparation we used recombinant soluble DR5 to neutralize TRAIL. The results of this experiment shown in Fig. 4 demonstrate that soluble DR5 blocks TRAIL-induced cell death while soluble TNF R1 does not.

To definitively identify the apoptotic cells as thyroid follicular cells, we used an antibody that is specific for CK 18, a marker for epithelial cells, but not fibroblasts (which commonly grow out of primary TEC cultures). Fig. 5A shows CK18 expression in 90.6% of untreated cells and 82.2% of TNFα and IL-1β pretreated cells in thyroid cultures which were treated with TRAIL for induction of apoptosis (shown in Fig. 5B). Fig. 5B displays the results of staining the same cells used in Fig. 5A with the M30 CytoDEATH® antibody that specifically detects apoptotic epithelial cells by binding to caspase-cleaved CK18. 10.7% of the cells treated with cytokines and TRAIL were both epithelial and apoptotic in this assay. This is compared to only 1.1% of the cells treated with inflammatory cytokines but not treated with TRAIL and <1% of the cells without inflammatory cytokine treatment regardless of TRAIL treatment. This documents that TRAIL induced apoptosis occurs in TECs. The addition of IFNγ to the other inflammatory cytokines in this assay caused a small increase in background death (from 1.1 to 1.8%, as was seen in most of our assays) but significantly reduced TRAIL specific apoptosis from 10.7% to 3.3%. The occurrence of IFNγ protection has been observed in ten separate experiments from primary tissues derived from different patients. Fibroblasts and vascular smooth muscle cells are common contaminants of TEC cultures and will overwhelm the epithelial cells over extended culture. Treatment of fibroblast or vascular smooth muscle cells derived from thyroid cultures with cytokines and TRAIL showed no cell death while remaining susceptible to Fas-mediated cell death (data not shown).

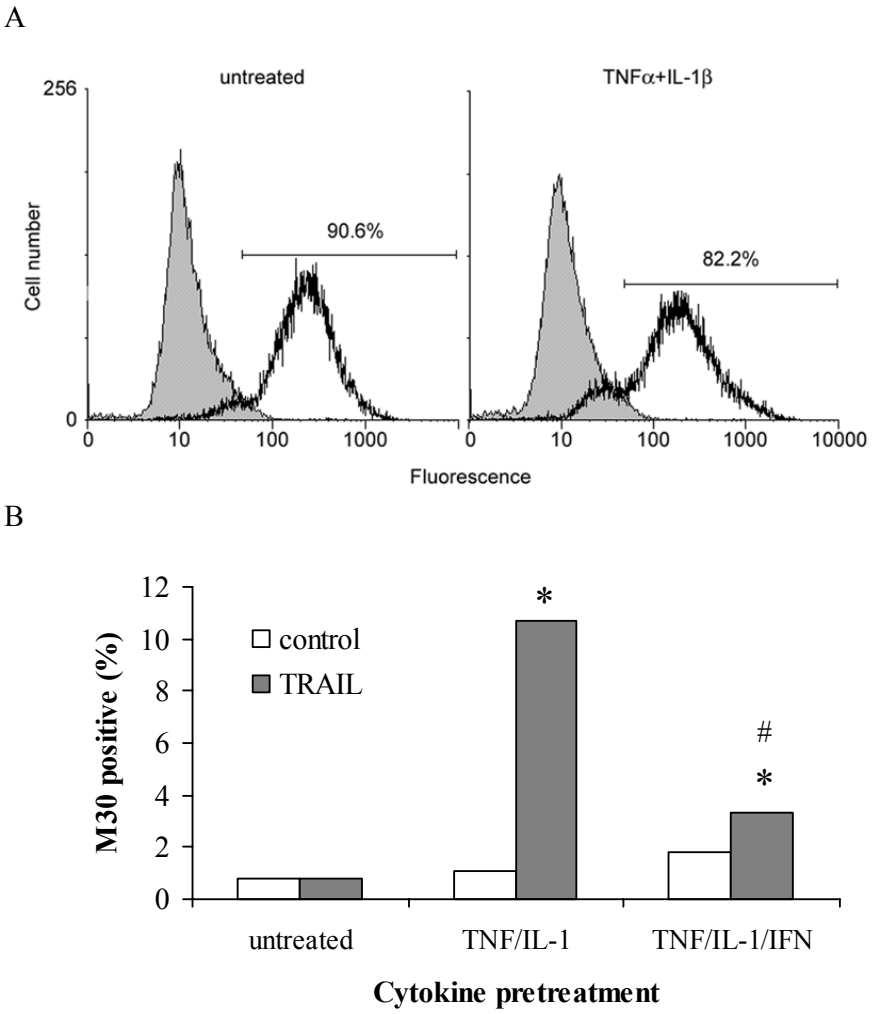


Figure 5. *TRAIL induction of apoptosis occurs primarily in TECs*.
(A) Flow cytometry analysis of TECs treated for 4d with cytokines prior to harvesting for staining and flow cytometry. The filled curve of each panel represents the fluorescence of the control Ab. The open curve represents the fluorescence of the CK18-specific Ab.
(B) Flow cytometry analysis of TECs treated as above with the addition of TRAIL 6h prior to harvesting of cells and staining with M30 CytoDEATH® Ab. Asterisks denote results of TRAIL treatment with $p < 0.0001$ when compared to cultures not treated with cytokines. # denotes $p < 0.0001$ when compared to the TRAIL and TNFα/IL-1β treated culture.

Several assays were performed to further characterize the phenomenon of inflammatory cytokine-induced TRAIL-mediated apoptosis in normal TECs. Fig. 6 shows a dose-response curve where TRAIL, in concentrations >50ng/ml, was capable of mediating the inflammatory cytokine induced cell death with maximum killing occurring at the highest concentration used (800 ng/ml). This range of concentrations is higher than that required for CHX-induced susceptibility to TRAIL-mediated apoptosis (Bretz 1999b).

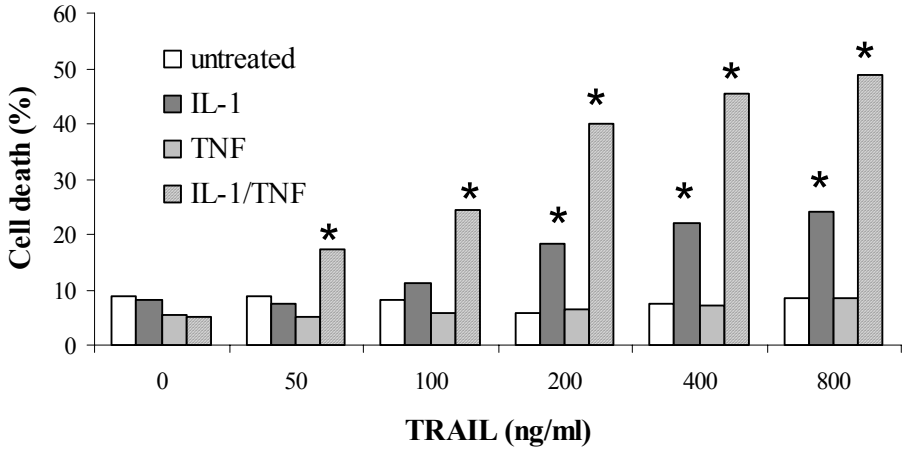


Figure 6. *Dose response of TRAIL-induced cell death in TECs*
TEC cultures were treated with the indicated cytokines and assayed for cell death as described for Fig. 1. Asterisks denote results of TRAIL treatment with $p < 0.0001$ when compared separately with both the control sample (no cytokines) and untreated (cytokines but without TRAIL).

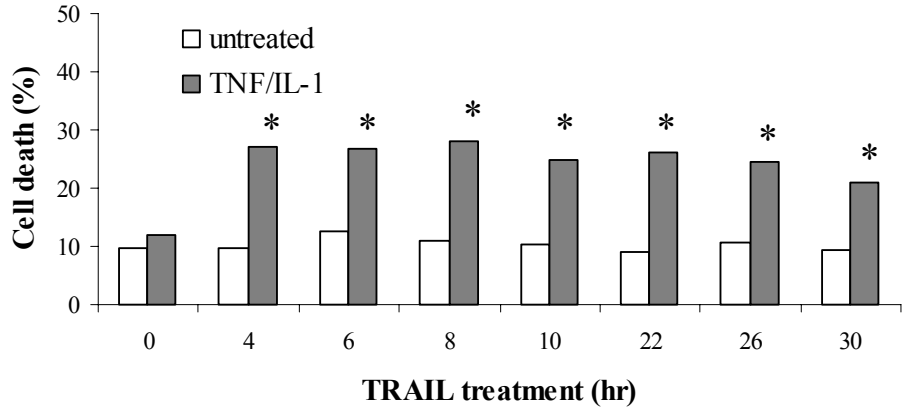


Figure 7. *Time course of TRAIL-induced cell death in TECs*
A time-course of TRAIL cytotoxicity was performed on TEC cultures pretreated for 4d prior to harvesting for staining and flow cytometry as described above. TRAIL (800ng/ml) was added at the indicated times prior to cell harvest. Asterisks denote results of cytokine treatment with $p < 0.0001$ when compared separately with both the control sample (0h TRAIL treatment) and untreated (TRAIL but without cytokines).

Fig. 7 shows that near maximum cell death occurs within 4h and that level of cell death is detectable for at least 18 more hours. This is likely due to rapid cell killing that reaches a limit at 4-8h. The dead cells then continue to remain demonstrable at later time points in this assay. The rapidity of killing is comparable to uninhibited Fas-

mediated killing in other systems (Itoh 1991, Luttman 1998) and in inflammatory cytokine treated thyroid cells (Bretz 1999a).

Annexin V staining of the outer leaflet of the plasma membrane was used to confirm that the dying thyroid cells were undergoing apoptosis. Fig. 8 shows that significant Annexin V staining of intact cells occurred between 2 and 4h. This also confirms the time course data in Fig. 7.

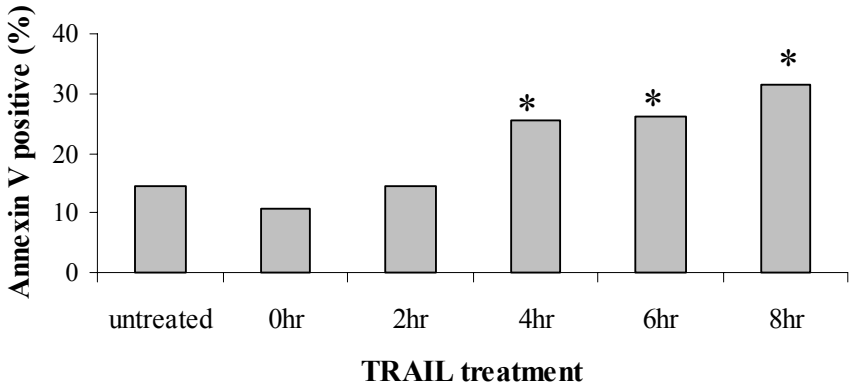


Figure 8. *Measurement of TRAIL-induced cell death in TECs by Annexin V staining*
Cell cultures were treated for 4d with TNFα/IL-1β, and TRAIL was added at the indicated time prior to cell harvest for staining and flow cytometry. Asterisks denote results of TRAIL treatment with $p < 0.0001$ when compared to untreated cultures. TRAIL was also added immediately prior to harvest (0h) to control for any apoptosis occurring after harvest but prior to flow cytometry quantitation. Only PI negative cells were used for quantitating Annexin V staining.

4.2. TRAIL receptor expression and regulation in thyroid epithelial cells

It was published that DR4 and DR5 mRNA is expressed in untreated normal primary TECs (Bretz 1999b). To determine whether regulation of expression of the TRAIL death receptors or decoy receptors may be involved in the control of thyroid cell apoptosis we performed RNase protection assays to determine relative mRNA expression levels in thyroid cell cultures treated with inflammatory cytokines. Fig. 9 displays an autoradiogram showing relative levels of expression of DR4, DR5, DcR1 and DcR2 in thyroid cell cultures after four days treatment with various combinations of inflammatory cytokines.

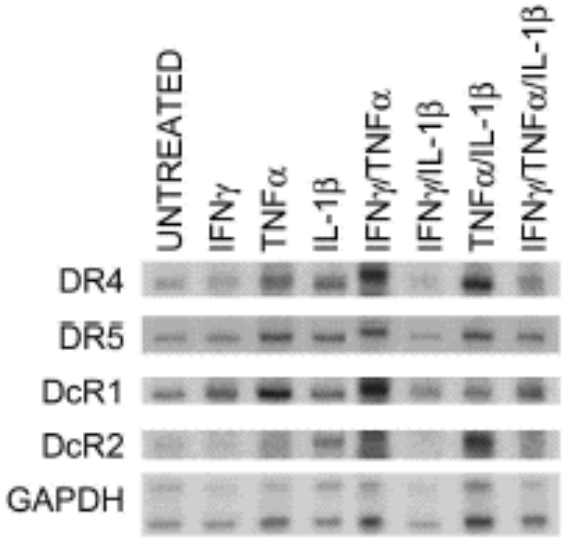


Figure 9. *Regulation of TRAIL receptors' mRNA expression in TECs by inflammatory cytokines*
RNase protection analysis was performed on mRNA isolated from TEC cultures treated for 4d with the indicated cytokines. This experiment is representative of results from five independent experiments each using cultures derived from different patient samples.

All four TRAIL receptor genes are expressed at the mRNA level regardless of cytokine treatment conditions. Also, all four genes appear to be regulated by certain combinations of cytokines, but changes in DR4 or DR5 mRNA levels do not correlate with conditions that promote TRAIL-mediated apoptosis (IL-1β or TNFα/IL-1β treatment). Interestingly, DcR1 mRNA expression is decreased by 50% by the combination of TNFα with IL-1β when compared to the untreated control (as measured by densitometry and corrected for mRNA loading by comparison with GAPDH levels) and is unchanged or increased by other inflammatory cytokines including IL-1β alone (50% increase). DcR2 mRNA expression, in conflict with its proposed function as an inhibitor of TRAIL-mediated apoptosis, increases dramatically under conditions of TRAIL susceptibility.

To further characterize the regulation of TRAIL receptors we performed western analysis on whole-cell lysates from TECs treated with inflammatory cytokines to determine relative levels of total protein expression. The results are shown in Fig. 10.

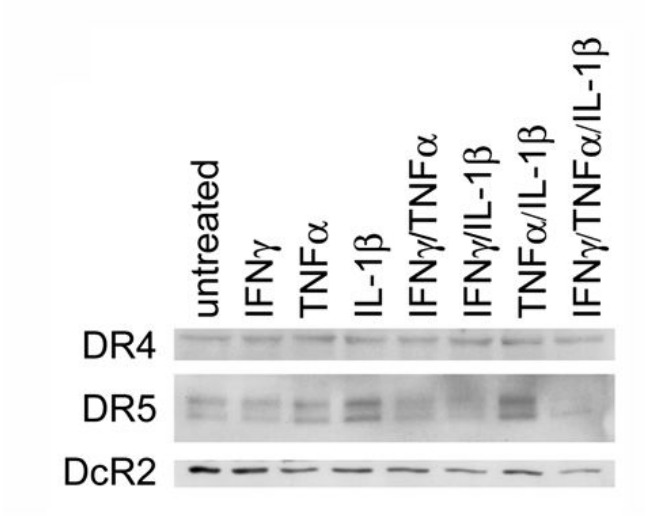


Figure 10. *Regulation of TRAIL receptors' expression in TECs by inflammatory cytokines*
Western analysis of TECs treated for 4d with the indicated cytokines prior to harvesting and probed with antibody specific for the indicated TRAIL receptors. This experiment is representative of results from five independent experiments each using cultures derived from different patient samples.

DR5 protein was found present under all conditions but was increased significantly by treatment with IL-1β alone and in combination with TNFα. DR5 was barely detectable after treatment with all three cytokines. This regulation coincides with sensitivity to TRAIL. DR4 and DcR2 were present under all treatment conditions but expression levels did not coincide with TRAIL sensitivity. DcR1 expression was difficult to interpret by western analysis due to low sensitivity of the primary antibody. To confirm these data, we immunostained to detect DR5 expression of inflammatory cytokine treated TECs in culture and observed the cells by light microscopy.

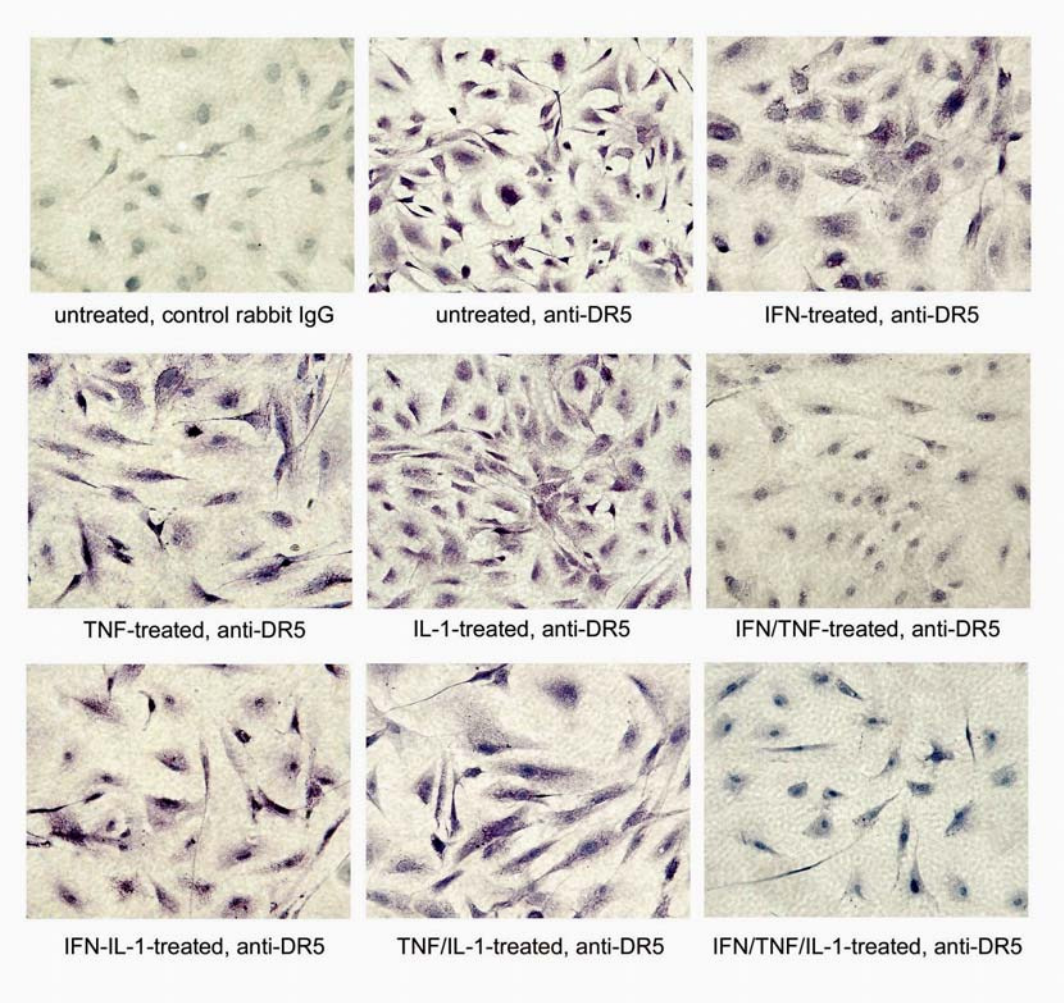
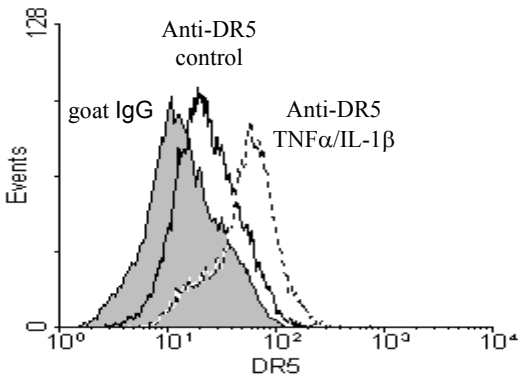


Figure 11. *Analysis of DR5 expression in TECs by immunocytochemistry*
Immunostaining of TECs treated for 4d with the indicated cytokines prior to staining with DR5 antibody or control rabbit IgG. Original magnification = 100X.

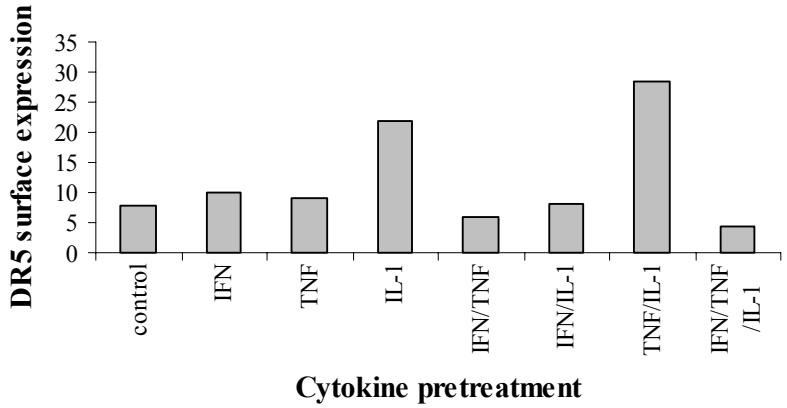
The results in Fig. 11 show high levels of DR5 expression in untreated cells and cells treated with each of the three cytokines alone or TNFα combined with IL-1β. In contrast, treatment with both IFNγ and TNFα or all three cytokines greatly reduces DR5 staining. The reduction in staining appears most significant in cytoplasmic regions while some staining is maintained in or around the nucleus. The decrease in DR5 in the IFNγ/IL-1β/TNFα treated cells may account for the resistance of these cells to TRAIL.

To provide a more definitive clarification of a role for TRAIL receptor regulation in TRAIL-mediated apoptosis of TECs we determined their cell surface expression after treatment with cytokines. Fig. 12 shows the results of flow cytometric determination of cell surface expression of DR5 and DcR1. DR5 and DcR1 were present in all untreated and cytokine treated cells. DR4 and DcR2 were undetectable (data not shown). This suggests that DR4 does not play a role in TRAIL-mediated apoptosis in TECs. DR5 cell surface levels increased significantly with IL-1β and more so with IL-1β/TNFα treatment and dropped to below untreated cell levels when IFNγ was added to either IL-1β or IL-1β/TNFα. DcR1 cell surface levels were suppressed by the IL-1β/TNFα treatment and returned to normal levels with the further addition of IFNγ. This is in agreement with its potential role as an inhibitor of TRAIL-mediated apoptosis. Each cytokine alone increased DcR1 expression. The greatest DR5 induction occurs under the same conditions as the greatest suppression of DcR1 and this correlates with the conditions of greatest sensitivity to TRAIL-mediated apoptosis.

A



B



C

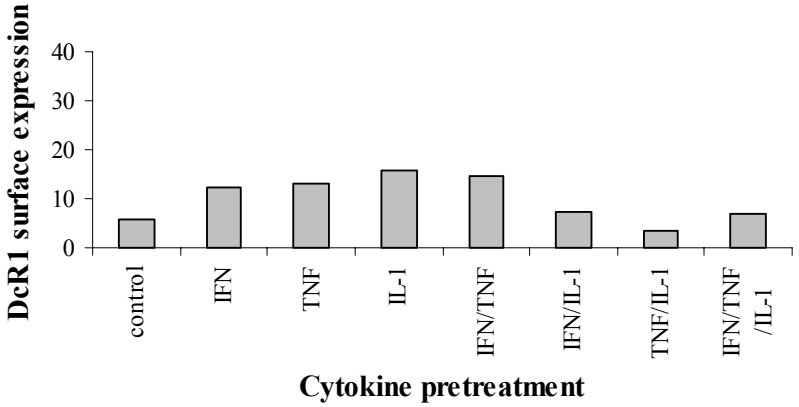


Figure 12. *Flow cytometric determination of cell surface expression of DR5 and DcR1*
TECs were treated with the indicated cytokines for 4d prior to harvesting for flow cytometry. 10 000 cells were assayed for each sample.
(A) A representative histogram of anti-DR5 antibody specific fluorescence for untreated cells (open curve, solid line) and TNFα/IL-1β treated cells (open curve, spotted line) is presented and compared to control goat IgG (filled curve).
(B) Anti-DR5 antibody specific mean fluorescence intensity is displayed after subtraction of mean fluorescence intensity of the control antibody.

(C) Anti-DcR1 antibody specific mean fluorescence intensity is shown after subtraction of mean fluorescence intensity of the control antibody.

To further define the role of subcellular location of TRAIL receptors in TRAIL-mediated apoptosis we performed confocal microscopy on cytokine treated TECs. Fig. 13 displays the results of this analysis. DR5 expression is present regardless of cytokine treatment and is mostly intracellular. In agreement with the flow cytometry data cell surface DR5 appears to increase with IL-1β/TNFα treatment and decrease with the further addition of IFNγ. DcR1 surface expression also shows agreement with the flow cytometry data as increased fluorescence is shown at the junctions of untreated and IFNγ/IL-1β/TNFα treated cells although most fluorescence is intracellular. Decreased DcR1 expression occurs after IL-1β/TNFα treatment.

4.3. DR5 transmits the apoptotic signal to IL-1β/TNFα treated thyroid cells

During the course of our investigations we determined that a DR5-specific polyclonal antibody produced in goat (R&D Systems lot # BVQ02) had the ability to act as an agonist for inducing apoptosis. This agonist antibody was specific for DR5 since soluble DR5 was able to block this activity while soluble DR4 and TNF R1 were not (data not shown). As shown in Fig. 14 the DR5 agonist antibody induces apoptosis in IL-1β/TNFα treated TECs and this activity is suppressed by the further addition of IFNγ. Soluble DR5 was able to neutralize the effects of this antibody while soluble TNF R1 was not, thus proving its specificity. This is identical to TRAIL-mediated apoptosis of these cells. This data along with the DR5 cell surface expression data strongly suggests that TRAIL acts through DR5 to induce apoptosis in TECs.

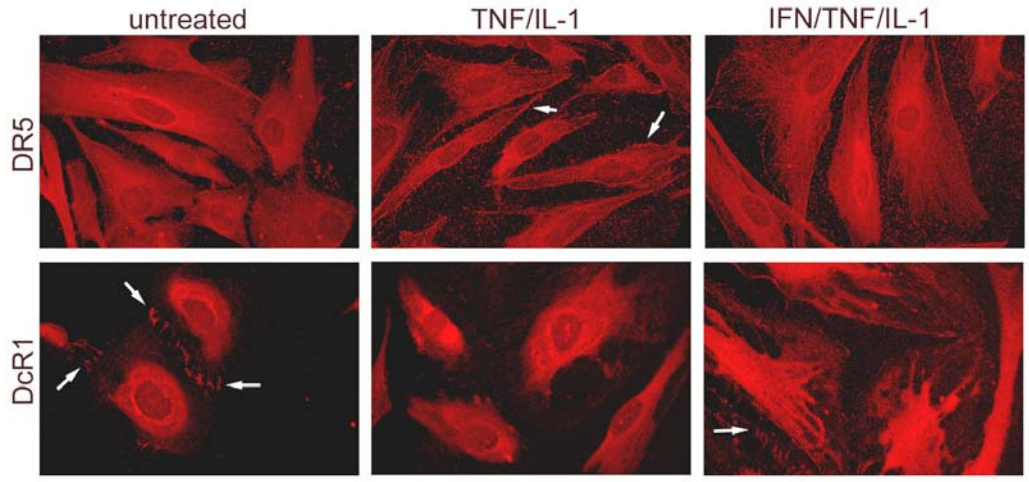


Figure 13. Laser scanning confocal microscopy of TECs stained for DR5 and DcR1 expression after 4d treatment with the indicated cytokines. Arrows indicate cell surface staining. Original magnification = 200X

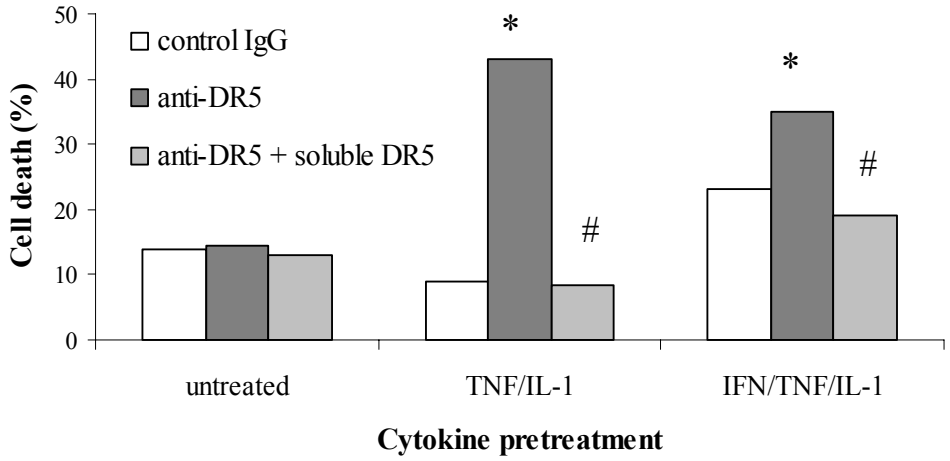


Figure 14. *Cell death in TECs is induced by a DR5-specific agonist antibody*
TECs pretreated with the indicated cytokines for 4d were treated with control goat IgG or goat anti-DR5 antibody with or without preincubation for 1h with soluble DR5. Results were quantitated as in Fig. 1. An asterisk denotes the results of anti-DR5 treatment with $p < 0.0001$ when compared to control IgG treated. The # symbol denotes the results of anti-DR5 + soluble DR5 treatment with $p < 0.0001$ when compared to anti-DR5 treatment alone with the same cytokine pretreatment.

4.4. TRAIL and TRAIL receptors are present in normal and thyroiditis glands

To determine the potential role for TRAIL-mediated apoptosis *in vivo* we endeavored to document the expression of TRAIL receptors in normal and diseased thyroids. Immunohistochemical staining of paraffin embedded thyroid tissue sections from patients with chronic autoimmune thyroiditis was compared staining with sections from normal thyroid gland. As shown in Fig. 15, all TRAIL receptors are present on TECs regardless of whether they are normal or involved in thyroiditis. A significant portion of the infiltrating lymphocytic cells in the thyroiditis tissue also express DR5 and DcR1. These results were confirmed using another antibody specific for a different epitope of DR5 (data not shown).

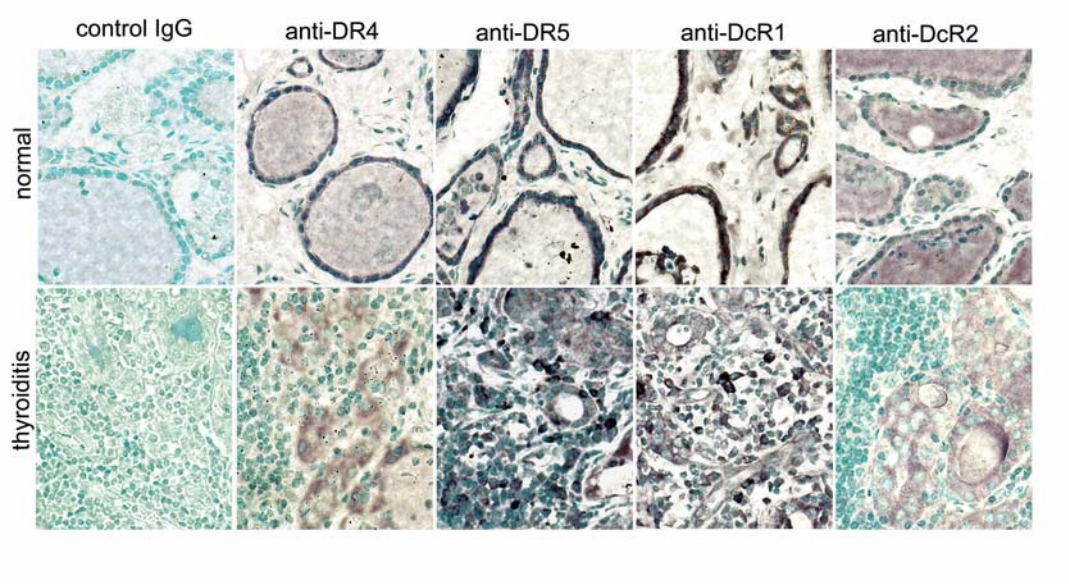


Figure 15. *Death receptor expression in the thyroid gland*
Immunohistochemical staining of thyroid sections was performed on samples from a normal thyroid (upper panels) and from a patient with chronic autoimmune thyroiditis (lower panels) using antibodies specific for the indicated TRAIL receptors and was compared to a rabbit control antibody (left panels). Original magnification = 100X.

It has been shown that TRAIL mRNA expression is increased by inflammatory cytokines in thyroid cell cultures (Bretz 1999b). Demonstrating the presence of TRAIL protein in the thyroid may suggest a mechanism for thyroid destruction in autoimmune thyroiditis. To determine whether TRAIL is expressed *in vivo* we immunohistochemically stained sections from the same thyroid tissue used in the experiments shown in Fig. 15. Fig. 16 demonstrates TRAIL expression in both normal follicular cells and oncocytic follicular cells from HT. The expression of TRAIL was markedly increased on TECs from thyroiditis, correlating with the data. However, no TRAIL expression was detected on infiltrating lymphocytic cells.

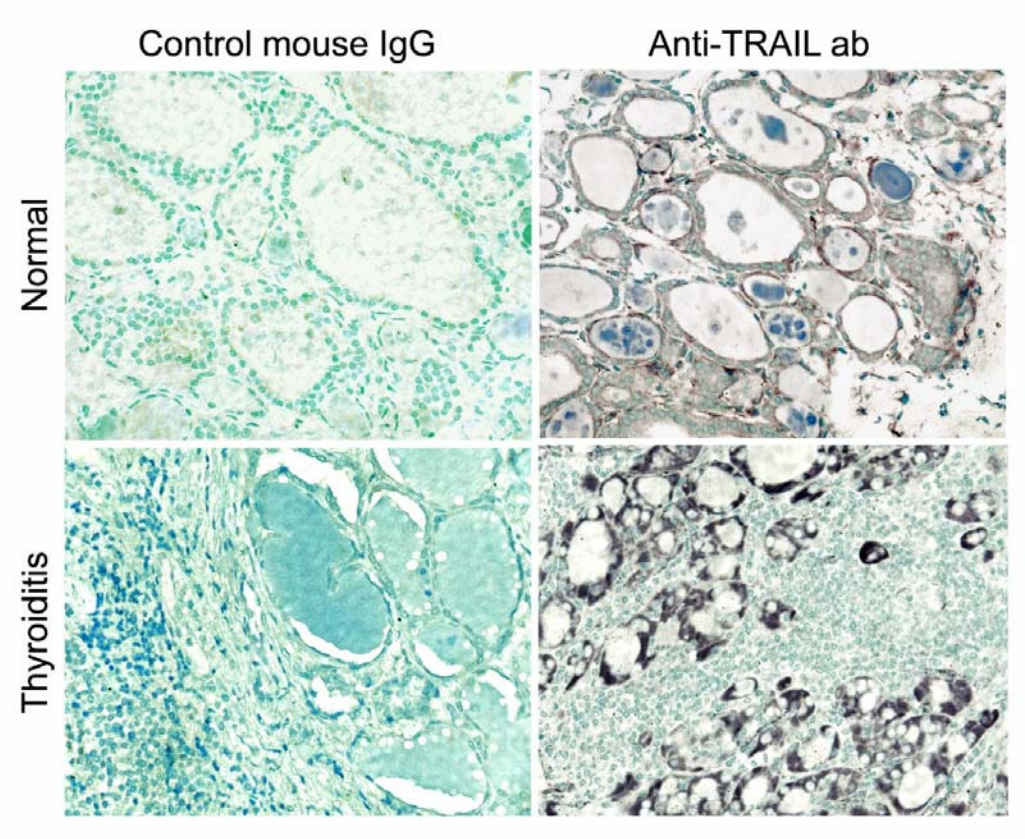
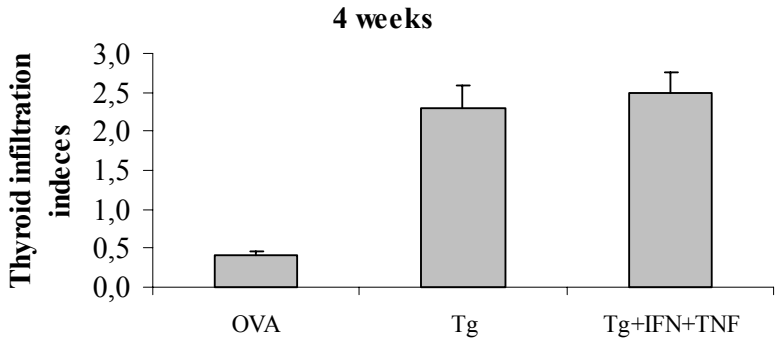


Figure 16. *TRAIL expression in the thyroid gland*
Immunohistochemical staining was performed on sections from the same samples used in Fig. 15 using an antibody specific for TRAIL (right panels) and a mouse IgG control antibody (left panels). Original magnification = 50X.

4.5. The combination of IFNγ and TNFα extends lymphocytic infiltration in murine EAT

To confirm our *in vitro* data about the essential role of inflammatory cytokines in regulating death susceptibility of thyroid cells, we modified a previously established EAT model. CBA/J mice were immunized with heterologous Tg in complete Freund's adjuvant to evaluate the effect of IFNγ and TNFα in murine EAT. IFNγ and TNFα, either singly or together, were injected into mice on week 3 after Tg immunization. On week 4 and 6 after initial immunization, thyroid glands were collected for histopathological studies.

A



B

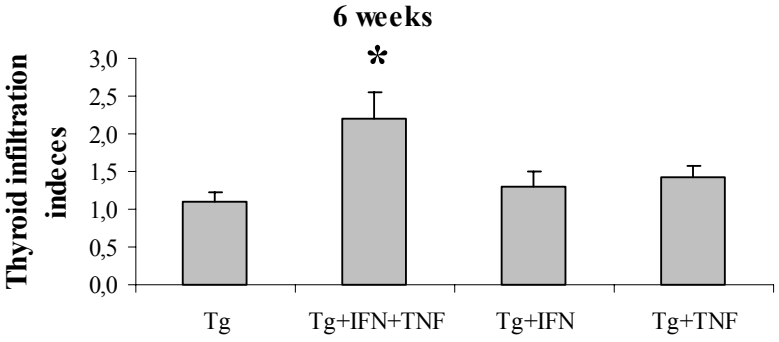


Figure 17. *Lymphocytic infiltration indeces of murine thyroid glands*
CBA/J mice were injected with the immunogen (OVA or pTg) and cytokines or BSA control, as described in *Materials and Methods*. Mice were sacrificed and the lymphocytic infiltration was quantified at 4 (A) and 6 (B) wk postimmunization. Values represent the mean±SD of 9-12 animals per group. *$p<0.01$ compared with Tg immunization alone.

As expected for this model, animals immunized with Tg showed mononuclear cell infiltration by 4 week postimmunization, but no infiltration was present in thyroids of mice injected with OVA. There also was no significant difference in infiltration between Tg-injected mice with or without cytokine treatment at this time point (Fig. 17A). In contrast, 6 week post-pTg immunization, thyroids from mice treated with pTg+IFNγ+TNFα showed markedly sustained mononuclear cell infiltration compared with thyroids from mice without cytokine treatment ($p < 0.01$; Fig. 17B).

The destruction of portions of the follicular architecture was observed in IFNγ/TNFα-treated mice (Fig. 18) but not in the animals immunized with either Tg alone or each cytokine singly.

4.6. Characterization of infiltrating cells

To identify the phenotype of cellular inflammation in the thyroid, we stained mice thyroid sections with CD45 antibody, which identifies leukocytes. Fig. 19 (upper right) identified the infiltrating mononuclear cells in the thyroid as primarily leukocytes. Further staining with CD4 and CD8 antibodies showed that both $CD4^+$ and $CD8^+$ T cells were present in thyroids from mice treated with pTg and IFNγ/TNFα, and these findings were similar to those observed in animals immunized with Tg alone (data not shown).

4.7. The combination of IFNγ and TNFα promotes thyrocyte destruction in EAT

Tg-induced autoimmunity in this model has not previously resulted in a destructive thyroiditis, although recent reports (Braley-Mullen 1998, Tang 2000) have

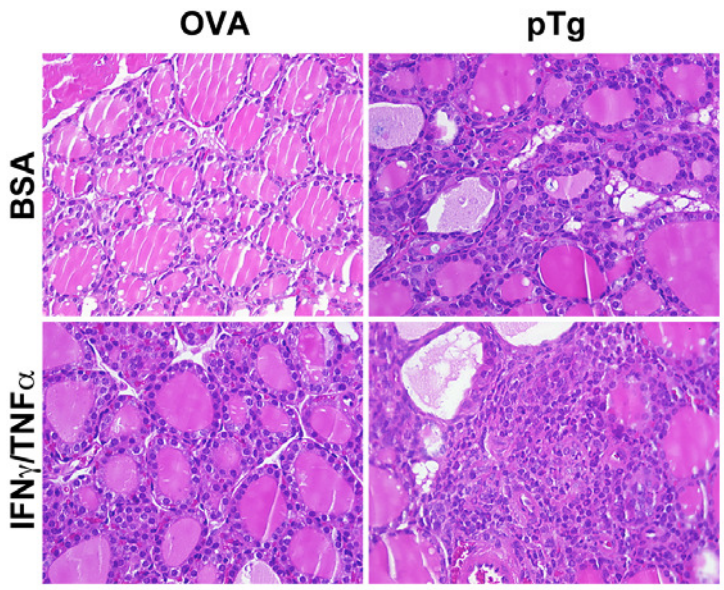


Figure 18. *Histology of thyroid glands*
Thyroid sections were prepared from mice at week 6 after initial immunization. Treatments of mice before sacrifice are indicated as follows: immunized with OVA (*upper left*); immunized with OVA then administered IFNγ/TNFα (*lower left*); immunized with pTg (*upper right*); immunized with pTg then given IFNγ/TNFα (*lower right*). Original magnification = 100X

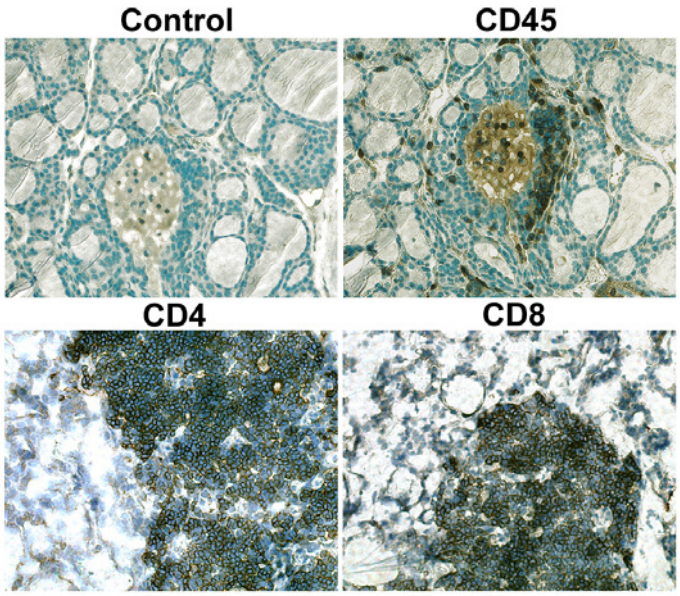


Figure 19. *Characterization of infiltrating cells*
Immunohistochemical staining of thyroid sections by CD4, CD8, CD45 and control antibodies. Thyroid sections are from mice immunized with pTg and treated with IFNγ/TNFα. Original magnification = 100X

demonstrated that cytokine treatments can alter the pathology of EAT from a lymphocytic to a granulomatous form.

To determine whether IFNγ and TNFα treatment specifically promotes thyrocyte destruction, thyroids from EAT mice were evaluated for cell death by in situ TUNEL staining. On week 6 after initial immunization, the number of apoptotic cells was markedly increased in the thyroids of EAT mice treated with the combination of IFNγ and TNFα, compared with mice without cytokine treatment. This was demonstrated by the number of TUNEL-positive cells shown in the right panel of Fig. 20.

It was suggested previously that alterations of the Fas pathway were involved in the ability of TNFα/IFNγ to facilitate apoptosis in thyroid cells (Bretz 1999a). Fas was expressed on the thyroid cell surface in our EAT mice, and the level of expression was not altered by cytokine treatment (data not shown). Therefore, we analyzed the function of the Fas pathway *in vivo* in only cytokine-treated mice by injecting an agonist anti-Fas antibody directly into the thyroid. The number of apoptotic thyroid epithelial cells was markedly increased in the thyroids of mice pretreated with IFNγ/TNFα compared with mice treated with Fas agonist alone (Fig. 21).

4.8. The IFNγ and TNFα treatment did not influence the humoral immune response to thyroglobulin in EAT

To ascertain whether the cytokine treatment and associated lymphocyte infiltration and thyrocyte apoptosis altered the humoral immune response, we determined the titer of antibodies to the immunogen pTg. Titers of anti-Tg antibodies were determined in sera from pTg- and OVA-immunized mice with or without cytokine treatment, and this was compared with preimmune serum.

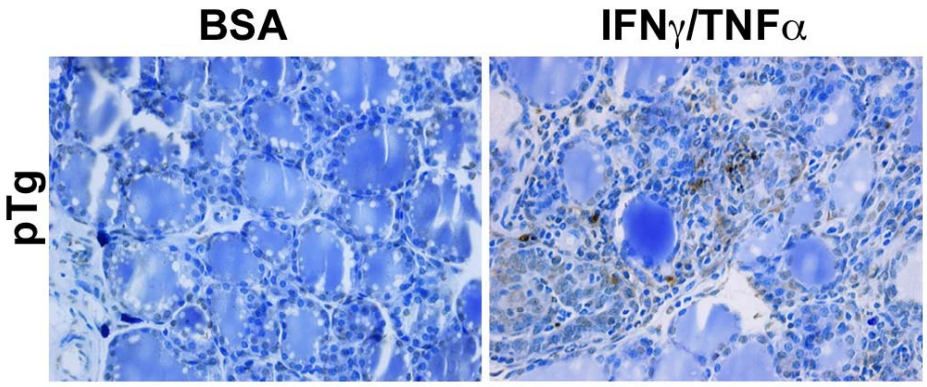


Figure 20. *Analysis of apoptosis in EAT mice*
In situ TUNEL staining of EAT thyroids to identify apoptotic cells. Sections from mice six weeks after initial immunization with pTg and treated with contrtol protein (*left panel*) vs those immunized with pTg and treated with IFNγ/TNFα (*right panel*). Original magnification = 100X

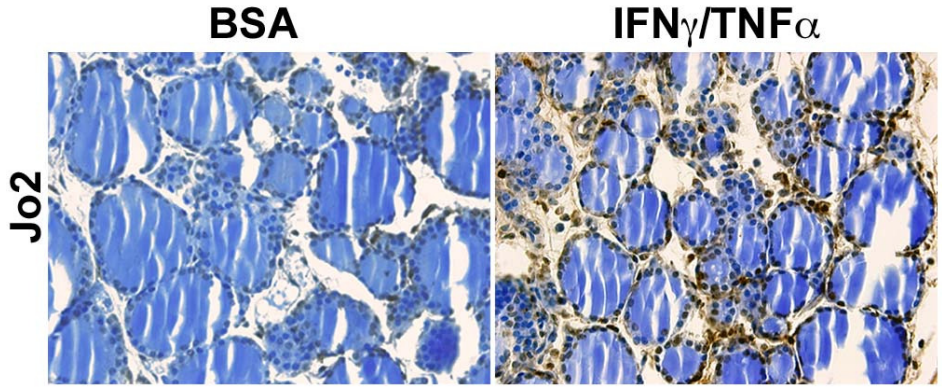


Figure 21. *Functional analysis of the Fas pathway in vivo*
Mice without immunization were treated with the agonist anti-Fas antibody (Jo2) injected directly into the thyroid and apoptosis was determined by TUNEL staining. Thyroid sections were prepared from mice injected with Jo2 alone (*left panel*) or with Jo2 after pretreatment witn both IFNγ and TNFα (*right panel*). Original magnification = 100X

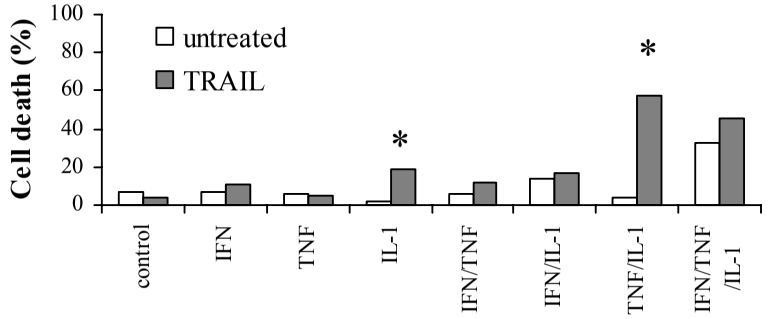
As expected, the titers of anti-Tg antibodies were significantly increased in all pTg-immunized mice, while control mice injected with OVA had no evidence of anti-Tg antibody regardless of cytokine treatment. However, the titers of anti-pTg antibodies from sera of pTg-immunized mice treated with or without cytokines did not differ significantly (data not shown).

IFNγ has a demonstrated role in modulating isotype switching (Snapper 1993), and to examine this effect in the EAT model, subclasses of antibodies to pTg were determined in sera from immunized mice treated with or without cytokine. The titers of IgG1 and IgG2a anti-Tg antibodies were similar in sera from Tg-immunized mice regardless of cytokine treatment (data not shown). We also examined the mouse serum antibodies for the ability to bind to purified mouse Tg using immunoblot analysis. Serum from pTg-immunized mice treated with or without cytokines produced staining of similar intensity to the mouse Tg band, which was identified using a commercial rabbit anti-Tg antibody (data not shown).

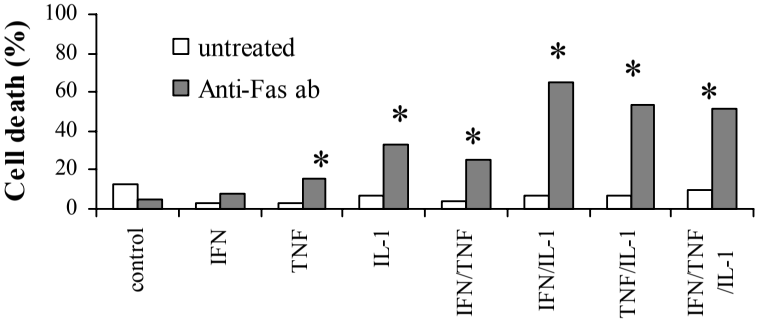
4.9. The majority of goiter-derived thyroid cells are resistant to TRAIL and/or FasL-mediated cell death after pretreatment with inflammatory cytokines

To investigate a potential role for aberrant apoptosis in the pathogenesis of nodular goiter we challenged primary thyroid cells from hyperplastic goiter nodules by inflammatory cytokines and treated by TRAIL or an agonist anti-Fas antibody. Similar to normal thyrocytes, thyroid cells from 17 goiter nodules were resistant to both death ligands under basal conditions (Fig. 22.). While normal thyroid cells could be sensitized to TRAIL and FasL by unique combinations of inflammatory cytokines, the majority of goiter-derived cells remained resistant to death ligands after cytokine pretreatment (Fig.22).

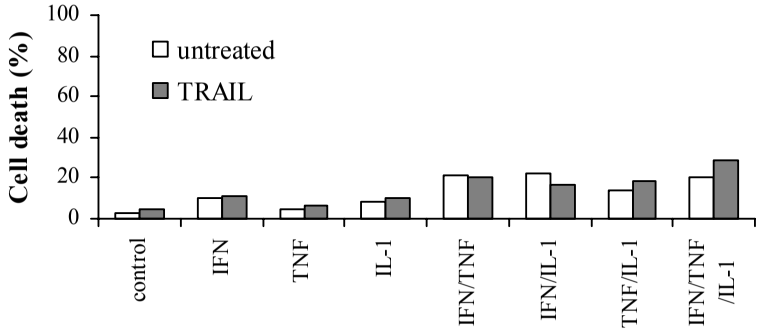
22A



22B



22C



22D

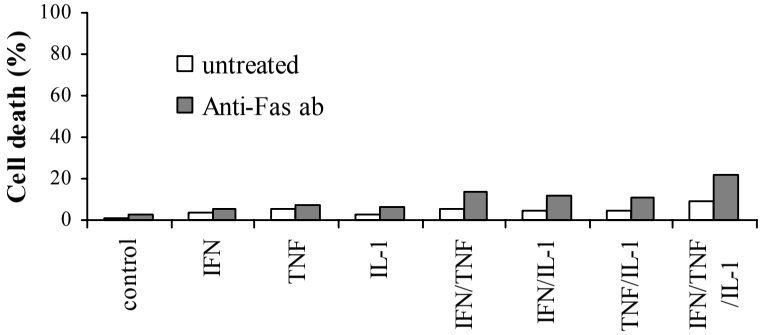


Figure 22. *Flow cytometric analysis of TRAIL- and Fas-mediated apoptosis after cytokine pretreatment in a normal (A and B) and a goiter sample (C and D)*
(A) Normal thyroid cells were exposed to 800 ng/ml TRAIL after four days pretreatment with the indicated cytokines. Cell death was determined by FDA and PI staining and 10 000 cells/sample were acquired by flow cytometry. Asterisk denotes conditions where $p < 0.0001$ compared with the control samples (no cytokines and cytokines without TRAIL).
(B) Normal thyroid cells were treated with 1 μg/ml agonist anti-Fas antibody (CH11) after four days pretreatment with the indicated cytokines. Cell death was assayed as above. * $p < 0.0001$ compared with the control samples (no cytokines and cytokines without CH11).
(C) Primary thyroid cells from a goiter nodule were treated and assayed as in Fig. 1A.
(D) Primary thyroid cells from a goiter nodule were treated and assayed as in Fig. 1B.
This experiment is representative of results from independent experiments using thyroid cells from ten normal thyroid tissues and seven resistant goiters.

The sensitivity of goiter cells to TRAIL- and Fas-mediated apoptosis after cytokine pretreatment was significantly decreased as compared to normal cells (Fig. 23A and B). The normal range was defined as mean$\pm$2SD of apoptosis rate in normal cells. According to their response to death ligands after cytokine pretreatment, the goiter cell populations were divided into sensitive (cell death within the normal range) and resistant (cell death below the normal range) groups. The susceptibility of cytokine pretreated goiter cells to TRAIL and anti-Fas antibody was diverse in several cases: seven goiters were resistant to death signaling through both pathways, five were resistant to TRAIL but sensitive to CH11, two were sensitive to TRAIL but resistant to CH11 and only three goiters were sensitive to both death ligands. In summary, the majority of goiter cells (14 of 17) remained resistant to at least one death ligand after pretreatment with inflammatory cytokines.

The comparison of TRAIL sensitivity in non-nodular and nodular thyroid cells from the same patients with multinodular goiter confirmed that the decreased death ligand sensitivity is characteristic for nodule-forming goiter cells (Fig. 24).

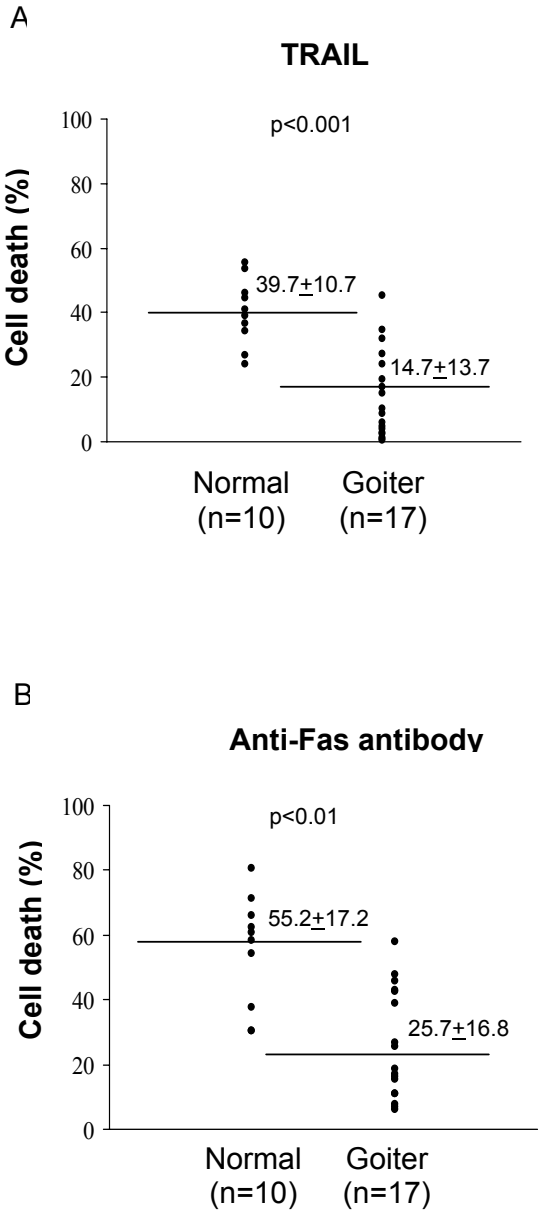


Figure 23. *Distribution of death receptor-mediated apoptosis after cytokine pretreatment in normal and goiter-derived cells*

(A) Primary thyroid cells from ten normal thyroid glands and seventeen nodular goiters were treated with 800 ng/ml TRAIL after four days pretreatment with TNFα/IL-1β. Cell death was measured by FDA and PI staining and quantitated by flow cytometry. 10,000 cells/sample were assayed, individual data points represent TRAIL-mediated percent cell death. Mean±SD is shown for each group.

(B) Normal and goiter-derived primary thyroid cells from the same patients as above were treated with 1 μg/ml anti-Fas antibody (clone CH11) after four days pretreatment with IFNγ/IL-1β. Cell death was assayed as above, individual data points represent Fas-mediated percent cell death. Mean±SD is shown for both groups.

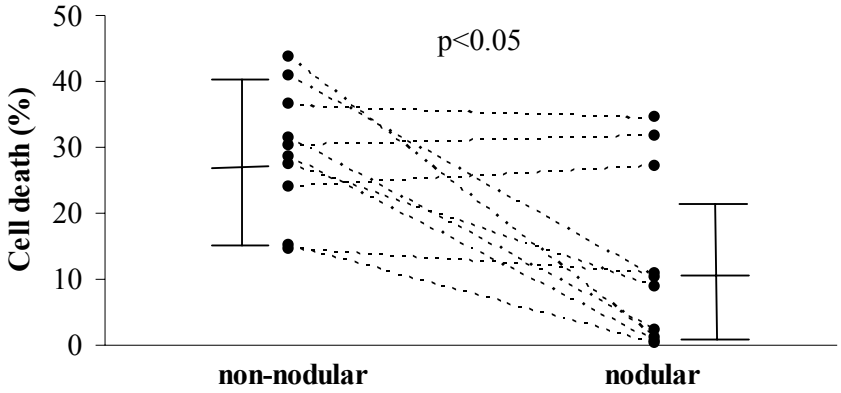


Figure 24. *TRAIL-mediated apoptosis in non-nodular and nodular thyroid cells from patients with multinodular goiter*
Primary thyroid cells derived from relatively normal, non-nodular parts of multinodular goiters and from well-defined nodules of the same patients (n=10). Data from the same patient are connected by dotted line. Cells were treated with 800 ng/ml TRAIL after four days pretreatment with TNFα/IL-1β. Cell death was measured by FDA and PI staining and quantitated by flow cytometry. 10 000 cells/sample were assayed, individual data points represent TRAIL-mediated percent cell death. Mean±SD is shown for each group.

4.10. Sensitivity to TRAIL-mediated apoptosis inversely correlates with goiter size

Goiter size was approximated by the weight of surgically removed thyroid as reported by pathological records. The mean weight of TRAIL sensitive goiters was significantly lower than the mean weight of the TRAIL resistant group ($p<0.05$). The TRAIL sensitive goiters were small (<25g) in every case, while the TRAIL resistant goiters formed a heterogeneous group, containing small and large goiters (reflected by the high SD value) (Fig. 25A). The susceptibility to anti-Fas antibody did not show a relationship with goiter size (Fig. 25B).

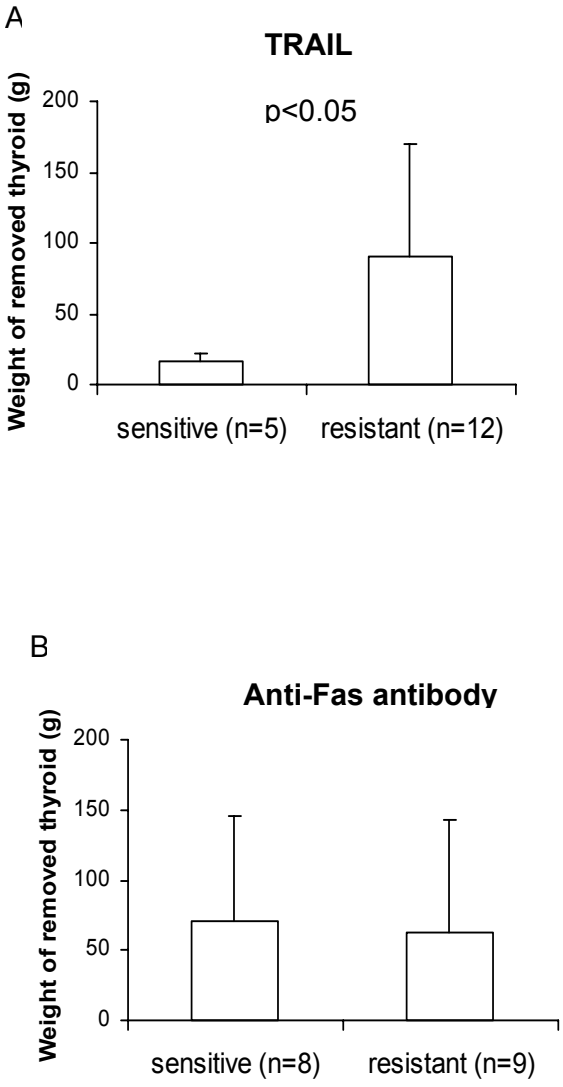


Figure 25. *Comparison of goiter size between TRAIL and FasL sensitive and resistant goiters*

(A) Goiter-derived thyroid cell cultures were divided into two groups according to their sensitivity to TRAIL after cytokine pretreatment. The normal range was defined as mean±2SD of apoptosis rate in normal cells. Cell populations responding to a lesser extent than this normal range, were categorized as TRAIL-resistant goiters. Cultures responding similar to normal cells, were categorized as TRAIL-sensitive goiters. Results are presented for each group as mean±SD weight of thyroid glands removed during thyroidectomy.

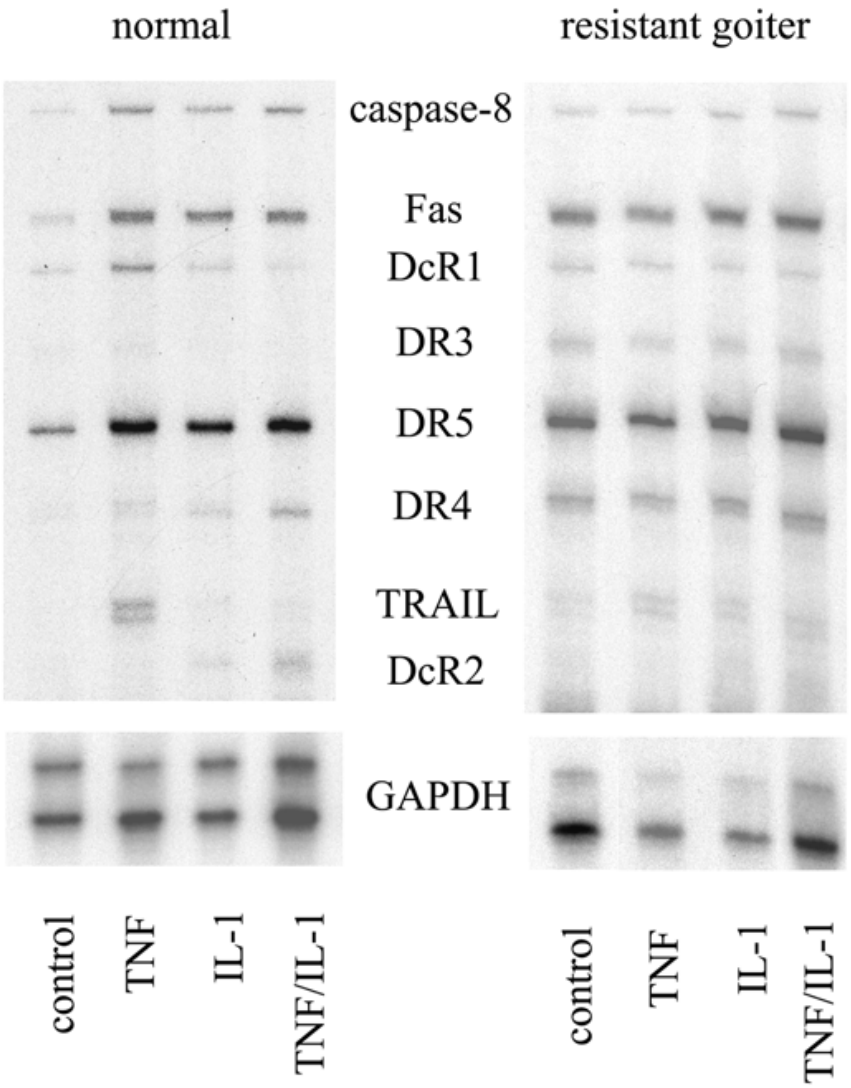
(B) Primary thyroid cell cultures from goiter nodules were divided into sensitive and resistant groups as above, according to their response to CH11. Data are presented as mean±SD weight of thyroid glands removed during thyroidectomy.

4.11. TRAIL receptor expression and regulation in goiter cells

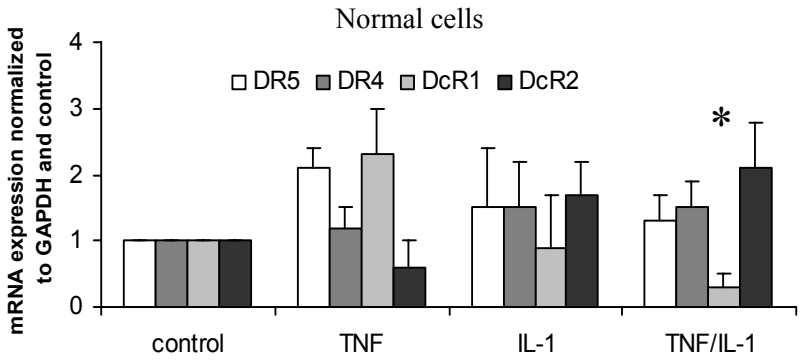
In order to define the mechanism of resistance to TRAIL-mediated cell death, the mRNA expression of TRAIL receptors was compared between five normal and three resistant goiter cell cultures after cytokine pretreatment, using RNase protection assays (Fig. 26). The expression of TRAIL receptors was moderately regulated by cytokines. TNFα treatment resulted in an approximate two-fold increase in DR5 and DcR1. A similar upregulation of DcR2 was found after TNFα/IL-1β treatment. Nevertheless, these upregulations did not correlate with enhanced TRAIL-mediated apoptosis since TNFα alone did not sensitize the cells to TRAIL and the expected result of DcR2 upregulation would be the protection of cells from TRAIL. Also of note, these changes did not differ between the normal and goiter groups. The mRNA level of DcR1 was significantly decreased by TNFα/IL-1β treatment in normal cells as compared to controls and goiters ($p<0.05$), however, further experiments excluded the involvement of DcR1 in goiter resistance (Fig. 28 and 29).

The death signal from TRAIL is transmitted by DR5 in normal thyroid cells. Despite the lack of DR5 mRNA regulation in the TRAIL-sensitive conditions, the total DR5 protein expression was upregulated by IL-1β and TNFα/IL-1β treatment and positively correlated with the sensitization of cells to TRAIL from five investigated normal thyroid tissues (Fig. 27). In sensitive goiter cells the upregulation of total DR5 expression was similar to that in normal cells, however in six resistant goiters these changes were diminished or absent (Fig. 27).

26 A


normal
resistant goiter
caspase-8
Fas
DcR1
DR3
DR5
DR4
TRAIL
DcR2
GAPDH
control
TNF
IL-1
TNF/IL-1
control
TNF
IL-1
TNF/IL-1

26B



26C

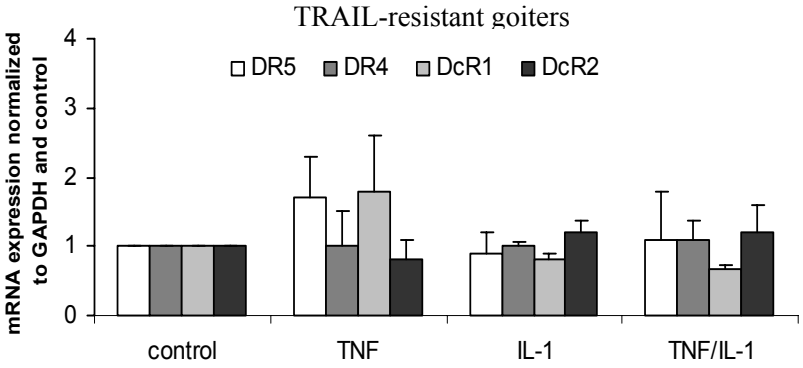


Figure 26. *Cytokine regulation of TRAIL receptor mRNA expression*
(A) RNase protection assay using the hAPO-3d template set was performed on 5 μg total RNA isolated from normal and goiter-derived thyroid cells after four days pretreatment with the indicated cytokines. This experiment is representative of results from independent experiments using thyroid cells from five normal thyroid tissues and three resistant goiters.
(B) The mRNA expression of TRAIL receptors was quantitated by densitometry and normalized to GAPDH signal and to TRAIL receptor expression in untreated cells. Data are presented as mean±SD of mRNA expression from five normal thyroid cell cultures. Asterisk denotes significant alteration of mRNA expression between the normal and the goiter group.
(C) The mRNA expression of TRAIL receptors was quantitated as above and normalized to GAPDH signal and to results in untreated cells. Data present the mean±SD of mRNA expression from three resistant goiters.

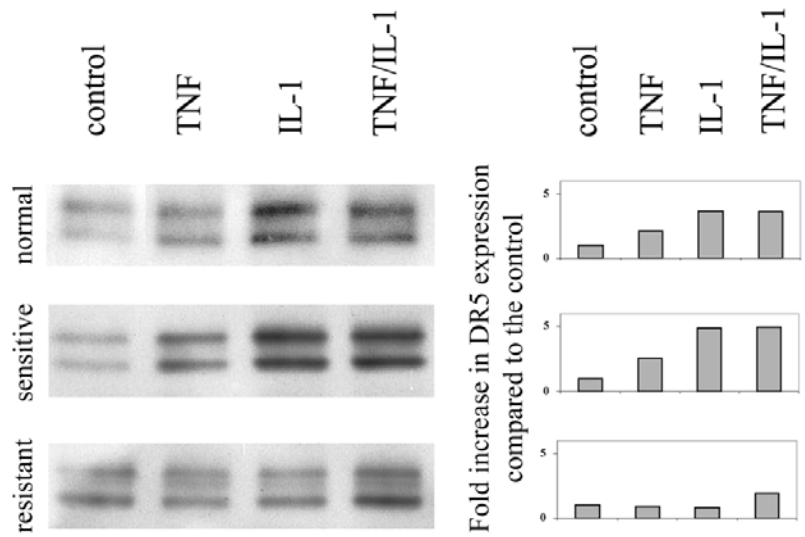


Figure 27. *Cytokine regulation of DR5 protein expression*
Western analysis of DR5 was performed from cell lysates of five normal, two TRAIL-sensitive and six TRAIL-resistant goiter cell cultures after four days pretreatment with the indicated cytokines. The autoradiograms were quantitated by densitometry and the DR5 expression after cytokine treatment was normalized to the result of untreated cells. Representative results are shown for each category.

The surface expression of TRAIL receptors was investigated in eight normal, four sensitive and six resistant goiter-derived cell populations. IL-1β and TNFα/IL-1β treatment uniformly resulted in a significant increase in the cell surface expression of DR5, both in normal and goiter cells, suggesting that the resistance of goiter cells to TRAIL was not caused by a lack of DR5 cell surface expression (Fig. 28). DR4 and DcR2 were not detected on either normal or resistant cells (data not shown). DcR1 surface expression was minimal in control cells and remained unchanged after TNFα/IL-1β treatment (Fig. 28).

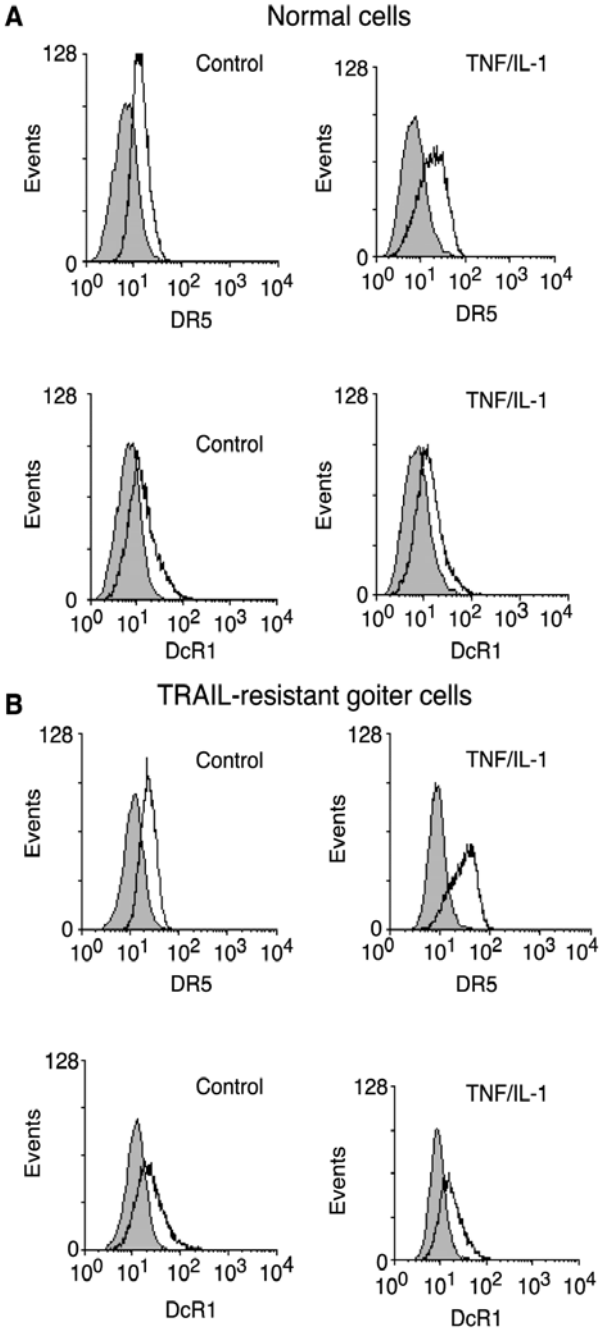


Figure 28. *Flow cytometric determination of DR5 and DcR1 cell surface expression*
(A) The analysis of DR5 and DcR1 cell surface expression was performed on eight normal thyroid cell populations with and without four days TNFα/IL-1β treatment. Representative histograms of DR5 and DcR1 antibodies are demonstrated (open curve) compared to control antibody (filled curve). (B) DR5 and DcR1 surface expression is presented as above in a TRAIL-resistant goiter cell population, which is representative of six resistant goiters.

4.12. TRAIL-resistant goiter cells are resistant to an agonist anti-DR5 antibody

In order to exclude a role for decoy receptors in mediating resistance to TRAIL, an agonist anti-DR5 antibody without cross-reactivity to DcR1 and DcR2 was used. The TRAIL resistant goiter cells were also resistant to the agonist anti-DR5 antibody (Fig. 29). This confirms that DR5 activation does not induce apoptosis in these cells, despite the presence of DR5 on the cell surface. The loss of DR5 signal transduction makes the participation of decoy receptors unnecessary to express goiter cell resistance.

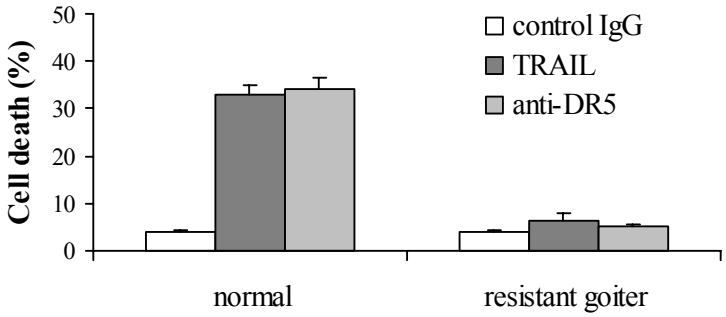


Figure 29. *The effect of an agonist anti-DR5 antibody in normal and TRAIL-resistant goiter cells*
Normal and goiter-derived cells were treated with 100 ng/ml goat control IgG, 100 ng/ml goat agonist anti-DR5 antibody or 800 ng/ml TRAIL after four days of TNFα/IL-1β pretreatment. Cell death was measured by FDA and PI staining and quantitated by flow cytometry. Data presented of mean±SD percent cell death of triplicate measurements, assaying 10 000 cells/sample.

4.13. Intracellular inhibitors of apoptosis in normal and goiter-derived cells

Cell cultures from five normal thyroid tissues and five TRAIL-resistant goiter nodules were compared for the expression of cFLIP and bcl-2 by Western blot analysis. No difference in baseline and cytokine-induced expression was observed for these proteins (data not shown). Members of IAP family were also unchanged (XIAP) or upregulated (cIAP-1, cIAP-2, NAIP) by pretreatment with TNFα and IL-1β (Fig. 30). These changes would be expected to increase protection instead of sensitization to death ligands.

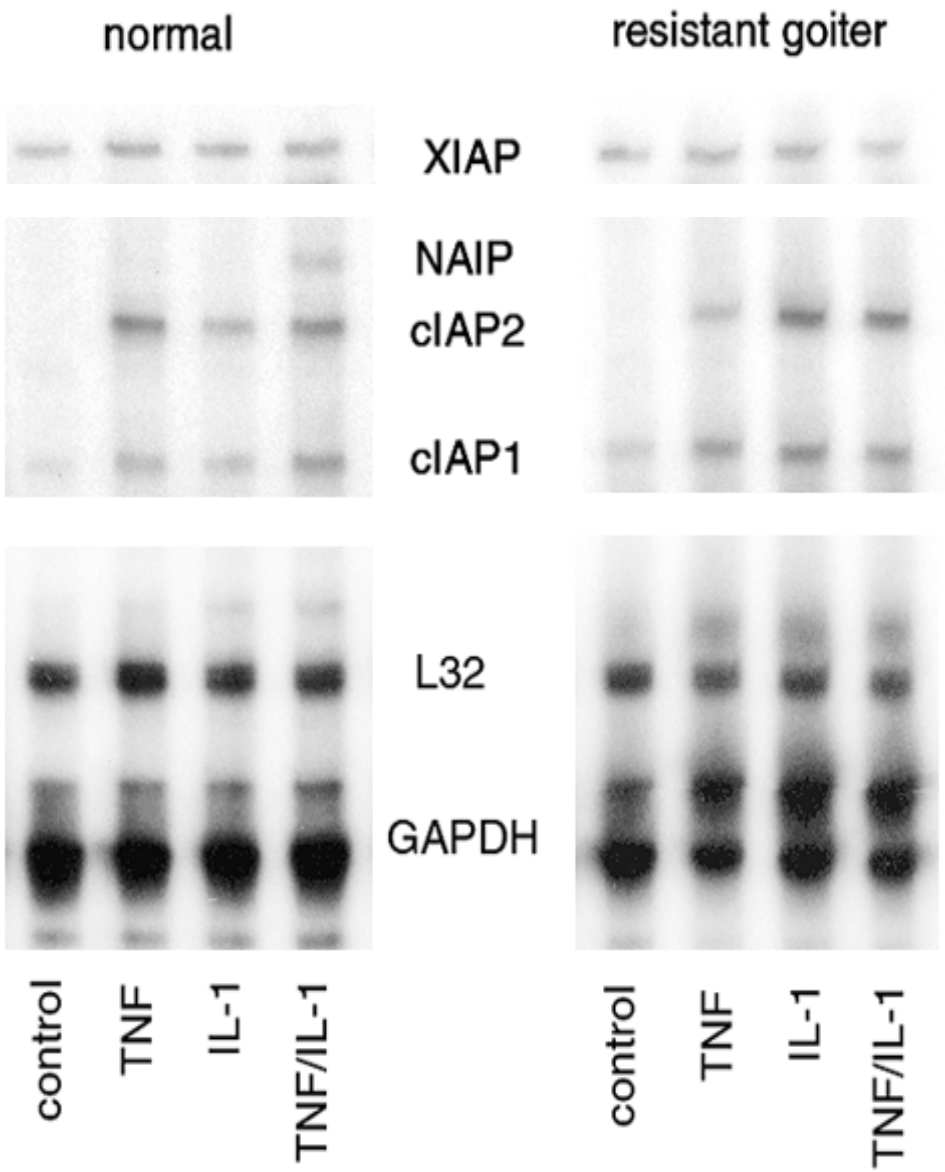
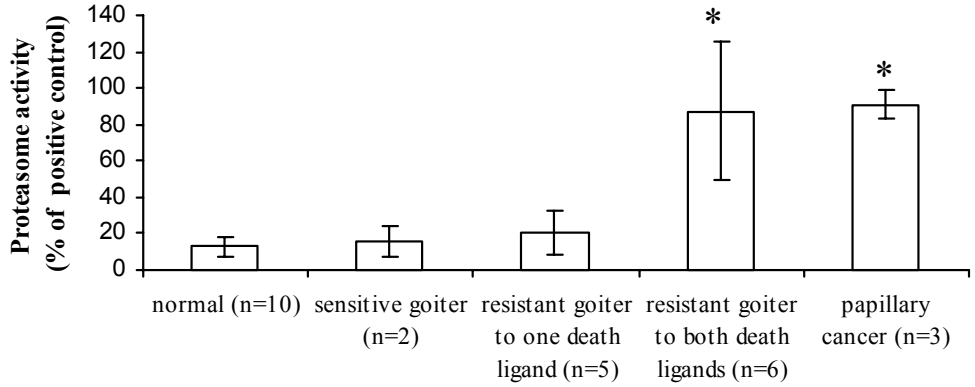


Figure 30. *Cytokine regulation of IAPs mRNA expression*
RNase protection assay using hAPO-5 template set was performed on 5 µg total RNA isolated from normal and goiter-derived thyroid cell cultures after four days pretreatment with the indicated cytokines. The results are representative of samples from three normal thyroid tissues and three TRAIL-resistant goiter nodules.

4.14. Proteasome activity positively correlates with the resistance to death ligands

Recent findings indicated that the proteasome system plays an important role in the regulation of apoptosis (Drexler 1997, Orlowski 1999, Kim 2001). Normal thyroid cells from ten individuals were characterized by low proteasome activity (mean±SD: 12.9+5.2%) (Fig. 31A). Lysates from six goiter cell cultures resistant to both death ligands demonstrated proteasome activity that was significantly increased over normal thyroid cells (87.2+37.9%), and was similar to the proteasome activity observed in papillary cancers (Fig. 31A). In contrast, death ligand sensitive goiters and goiters resistant to only one of the two death ligands showed low proteasome activity, similar to normal cells (Fig. 31A). The proteasome activity was also low in thyroid cells derived from the non-nodular part of multinodular goiters (data not shown). The treatment with proteasome inhibitor lactacystin restored sensitivity of goiter cells to TRAIL-mediated cell death (Fig. 31B). Restoration of TRAIL susceptibility in these cells by proteasome inhibition was confirmed by another inhibitor, MG132 (data not shown).

A



B

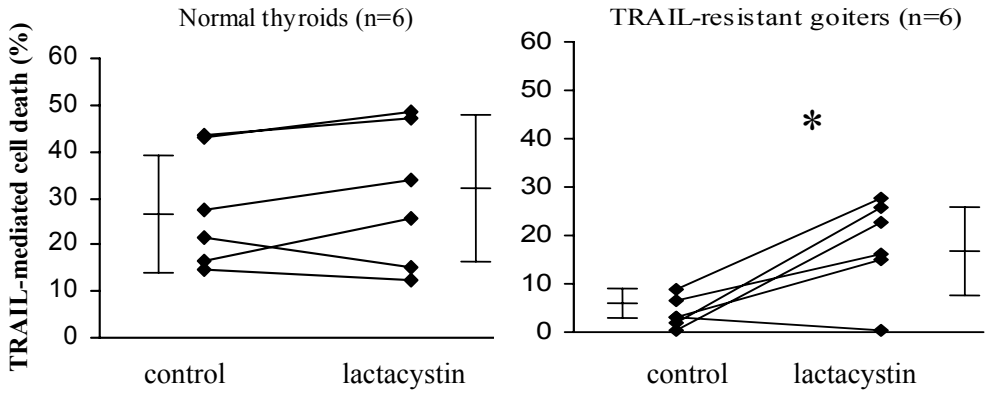


Figure 31. *Proteasome activity in thyroid cells and the effect of proteasome inhibition on TRAIL susceptibility*

(A) The proteasome activity of thyroid cells was measured by the hydrolysis of a fluorogenic substrate Suc-LLVY-MCA. The individual values were measured in triplicates with SD of <5% for all data points and standardized to the hydrolysis of substrate by 5 μg recombinant proteasome as a positive control (100 %). Results are presented as mean+SD of proteasome activity in the indicated number of patients, grouped according to the histological diagnosis and susceptibility to death ligands. The proteasome activity in papillary cancers is shown for comparison. *$p<0.05$.

(B) Normal and goiter-derived thyroid cells were pretreated for four days by TNFα/IL-1β and incubated with 800 ng/ml TRAIL with or without 1 μM lactacystin for 5 h. Cell death was measured by FDA and PI staining and 10 000 cells/sample were assayed by flow cytometry. Data presented as percent cell death with or without lactacystin. *$p<0.05$ (Student's t-test).

4.15. Establishment of a SCID mouse model to investigate engrafted human thyroid tumors

As DTC is believed to be influenced by anti-tumor immunity, an animal model was warranted to study the interaction of human immune system and tumor tissue. The engraftment of thyroid tumors into SCID mice was successful in every case. The growth rate was highly dependent on the histological type of thyroid cancer, with the highest rate seen in anaplastic carcinoma. By week 16, the xenografts from adenoma doubled in size, the size of papillary cancer tissues was 2.5+ 0.6 times, follicular and medullary cancer 3.2+0.5 times, anaplastic cancer 10 times in diameter. In anaplastic cancer grafts large cystic parts were observed which were not present in the original tumor at the time of surgery. The adjacent organs of mice were not infiltrated with human cancer cells, except in the case of anaplastic cancer, which was locally invasive. Distant metastasis did not occur. Immunohistochemical results of DTCs before transplantation are summarized in Table 2.

Table 2. Immunohistochemistry of differentiated thyroid cancers before transplantation

	CD20	CD3	CD8	CD45RO	HLA-DR	CD68
PC1	+	+	+	+	+	+
PC2	+	+	+	+	+	+
PC3	+	+	+	+	+	+
PC4	+	+	+	+	+	-
PC5	+	+	+	+	+	+
FC	+	+	+	+	-	+

PC: papillary cancer, FC: follicular cancer

Light-microscopic photomicrographs of the thyroid cancer tissues before transplantation are shown in Fig. 32. Before transplantation all DTCs were infiltrated with B and T lymphocytes (Fig. 32A and B). Macrophages were present in all (Fig. 32C) but one papillary cancer. HLA-DR expression on tumor cells was detected in every papillary cancer. Both strong HLA-DR expression and regions with less marked expression were

present in the same tumor (Fig. 32D). No HLA-DR expression was seen in the single follicular cancer studied (not shown). Immunohistochemical studies of the explanted grafts are summarized in Table 3.

Table 3. Immunohistochemistry of explanted differentiated thyroid cancer grafts

	CD20	CD3	CD8	CD45RO	HLA-DR	CD68	Tg
PC1A	-	-	-	-	-	-	+
PC1B	-	-	-	-	-	-	+
PC2A							
PC2B	-	-	-	-	+	-	+
PC3A	+	+	+	+	+	-	+
PC3B	-	-	-	-	-	-	+
PC4A	-	-	-	-	+	-	+
PC4B	-	-	-	-	+	-	+
PC5A	-	-	-	-	-	-	-
PC5B	-	-	-	-	-	-	+
FCA	-	-	-	-	-	-	+
FCB	-	-	-	-	-	-	+

PC: papillary cancer, FC: follicular cancer, A: reconstituted with peripheral blood mononuclear cells, B: not reconstituted, Tg: thyroglobulin, PC2A graft was not investigated because of the disappearance of tumor.

Light-microscopic photomicrographs of the thyroid cancer tissues after explantation are demonstrated in Fig. 33. After 16 weeks of growth in mice, the histological appearance of transplanted tumors proved to be unchanged, except one case where an anaplastic cancer arose from a papillary cancer (PC5A in Table 3, Fig. 33A). The original papillary structure remained in the mouse B. (PC5B in Table 3, Fig. 33B). The lack of thyroglobulin expression and aggressive growth characterized the anaplastic graft. In one case from group A the papillary cancer gradually decreased in size and disappeared, while its counterpart in group B showed continuous growth, doubling in diameter by week 16 (PC2A and B in Table 3). The tumor infiltrating lymphocytes (TILs) disappeared from all but one graft. However, in one case from group A (PC3A in Table 3) lymphocytes were detected in the tumor 16 weeks after transplantation (Fig. 33C). In this case (PC3) the 4 mm implant increased to 6 mm in mouse A (PBMCs reconstituted)

versus 10 mm in mouse B (not reconstituted). In the remaining cases no lymphocytic infiltration and no difference in the growth rate of the grafts was detected. Macrophages were absent in the explanted tissues at week 16. HLA-DR expression vanished from the papillary tumor cells in conjunction of the disappearance of lymphocytes in 3 cases. In PC3A (Table 3), the small amount of lymphocytes were enough to stimulate HLA-DR expression while its counterpart (PC3B in Table 3) was found to be negative. HLA-DR expression proved to be constitutive in 2 cancers (PC2B and PC4A and B in Table 3), namely HLA-DR expression was present in the lack of lymphocytes (Fig. 33D). The single follicular cancer originally was and remained negative for HLA-DR antigens in both mice A and B.

Human IgG levels at the end of the study (16 weeks after transplantation), were 2.96+3.63 and 0.78+1.32 mg/ml in groups A, and B respectively. The difference between groups A and B failed to reach statistical significance.

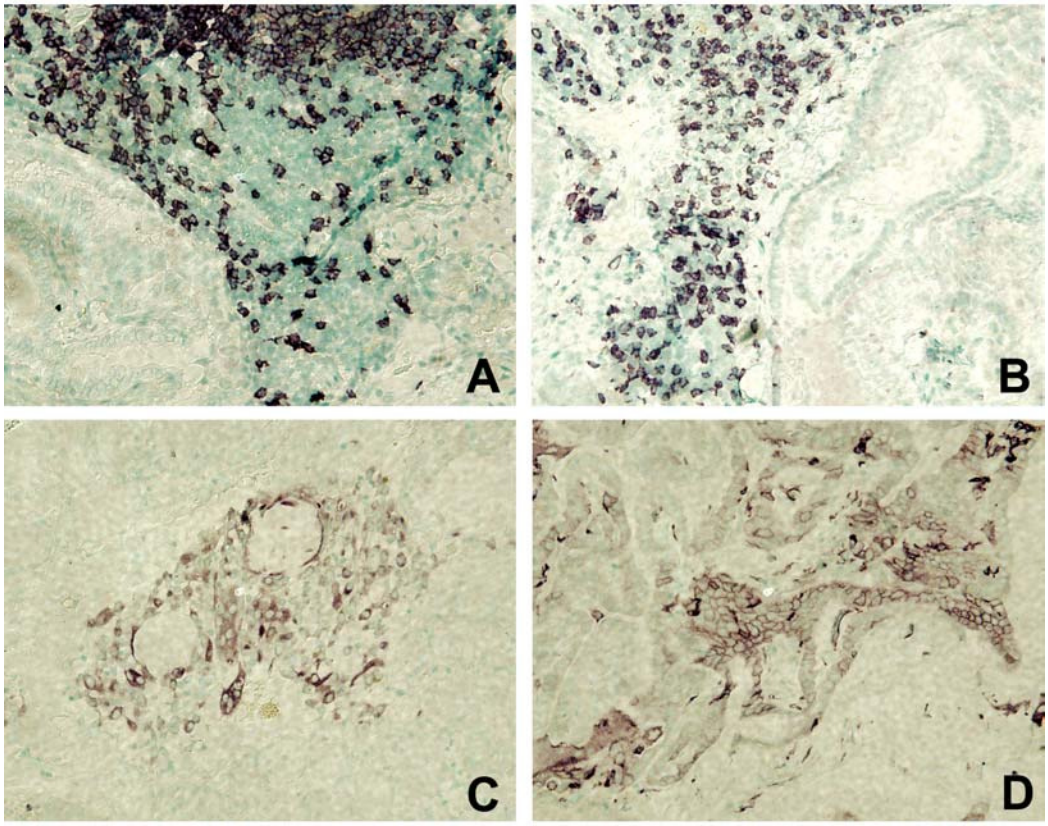


Figure 32. *Light-microscopic photomicrographs of the human thyroid cancer tissues before transplantation*
(A) B lymphocytes around papillary cancer (anti-CD20/immunoperoxidase)
(B) T lymphocytes around papillary cancer (anti-CD8/immunoperoxidase)
(C) Macrophages in papillary cancer (anti-CD68/immunoperoxidase)
(D) HLA-DR expression in papillary cancer (anti-HLA-DR/immunoperoxidase)
Original magnification = 50X

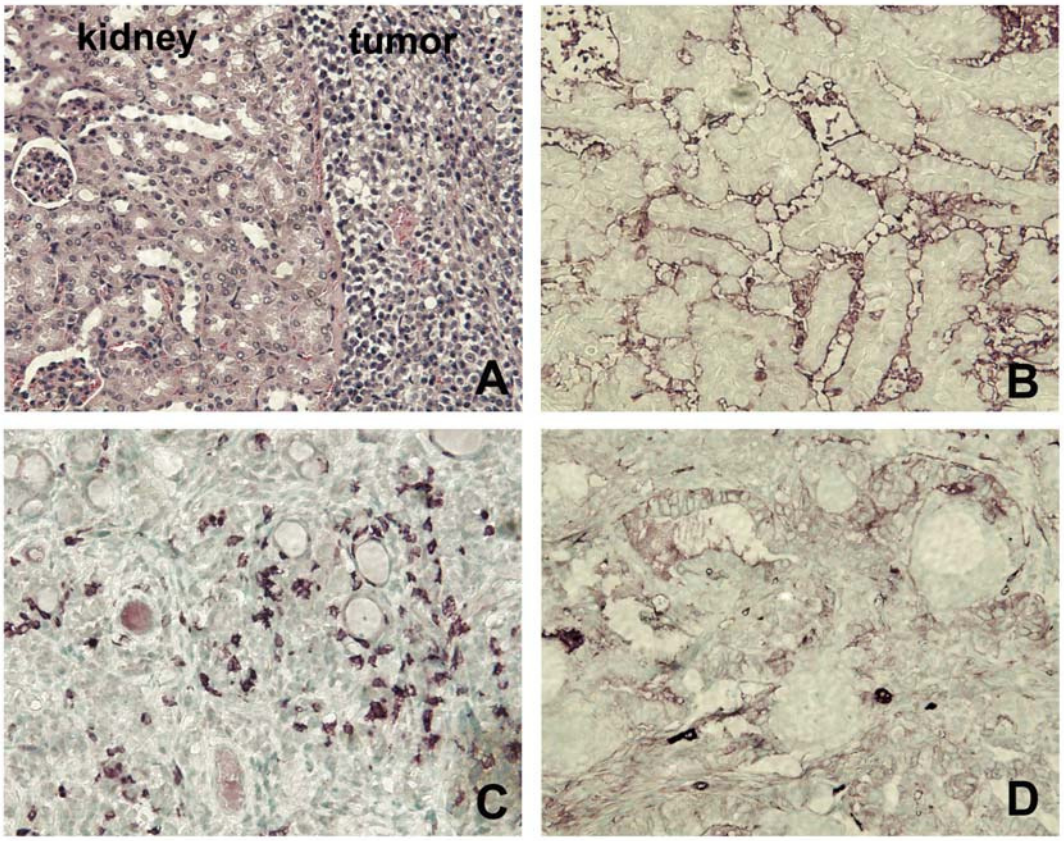


Figure 33. *Light-microscopic photomicrographs of the human thyroid cancer tissues after 16 weeks of growth in mice*
(A) Transplanted anaplastic cancer graft (arising from papillary cancer), invasive growth pattern, the tumor tissue reached the kidney of the mouse (H&E)
(B) Transplanted papillary cancer graft in SCID mouse, reserved thyroglobulin expression (anti-thyroglobulin/immunoperoxidase)
(C) T lymphocytes in transplanted papillary cancer graft (anti-CD8/immunoperoxidase)
(D) Constitutive HLA-DR expression in transplanted papillary cancer graft in the lack of lymphocytes (anti-HLA-DR/immunoperoxidase)
Original magnification = 50X

5. DISCUSSION

5.1. Inflammatory cytokine regulation of TRAIL-mediated apoptosis in human thyrocytes

There is general agreement that aberrant apoptosis of thyroid follicular cells is involved in autoimmune thyroid diseases and in the development of hyperplasias and neoplasias (Baker 1999). However, the exact mechanisms that cells use to signal apoptosis in thyroid disorders are unclear. Normal human thyroid cells *in vitro* are resistant to Fas- and TRAIL-mediated apoptosis (Arscott 1997, Bretz 1999b). It was shown that IFNγ combined with TNFα and/or IL-1β pretreatment of TECs could provide death susceptibility to Fas-mediated apoptosis (Bretz 1999a). The data presented here demonstrate that TRAIL-mediated apoptosis of TECs is also regulated by inflammatory cytokines. IL-1β alone and in combination with TNFα induces sensitivity to TRAIL, while the further addition of IFNγ makes these cells resistant to apoptosis induced by TRAIL. This is the first reported demonstration of IL-1β induction of TRAIL sensitivity and inhibition of TRAIL sensitivity by IFNγ. This pattern of TRAIL sensitivity correlates with levels of cell surface expression of TRAIL receptors. IL-1β/TNFα enhancement of DR5 surface expression provides a mechanism for TRAIL sensitivity. IFNγ counteracts this activity by suppressing DR5 surface expression, thus preventing apoptotic signal transduction. We have further documented that the signal is mediated through DR5 by the use of DR5 specific agonist antibody. These data are the first to demonstrate the induction of cell surface DR5 by IL-1β alone or in synergy with TNFα. This is also the initial demonstration of regulation of DcR1 cell surface expression levels by any treatment of any cell type especially primary cells. We also

show for the first time the *in vivo* presence of TRAIL and its receptors in the thyroid gland in both normal follicles and follicles undergoing autoimmune destruction.

Many articles have been published describing an apparent lack of correlation of TRAIL receptor expression (both death and decoy receptors) with susceptibility to TRAIL (Griffith 1998b, Griffith 1999, Frank 1999, Kim 2000, Jo 2000, Leverkus 2000, Mitsiades 2000, Wendling 2000, Zhang 2000b). It has been suggested that either downstream signal inhibitors are involved or endogenous levels of decoy receptors are not involved in the regulation of TRAIL-mediated apoptosis. Our results suggest that these inconsistencies are likely due to simply measuring mRNA or total protein, which do not reflect the functional form of the receptor on the cell surface. Others have shown constitutive surface expression of TRAIL death receptors on cells that were not susceptible to TRAIL (Kothny-Wilkes 1998). This suggests the possibility of an intracellular inhibitor of the TRAIL pathway. Our data regarding inflammatory cytokine regulation of TRAIL-induced apoptosis does not rule out the possibility of an intracellular inhibitor of this pathway. We have demonstrated in TECs that cytokine regulated apoptosis was not due simply to an increase in total expression of TRAIL death receptors. This agrees with previous findings that showed inhibition of protein synthesis in these cells could also enable the TRAIL pathway, possibly through selective loss of a labile inhibitor (Bretz 1999b). This inhibitor may act by preventing release of cytoplasmic DR5 to the cell surface. Expression levels of known inhibitors of apoptosis (bcl-2, bcl-X, XIAP, NAIP, cIAP-1, cIAP-2 and cFLIP) in our system are not regulated in a manner that correlates with susceptibility to TRAIL, neither does expression of pathway components caspase-8, caspase-10, caspase-3 and FADD (unpublished data).

DcR1 is a TRAIL decoy receptor that when overexpressed has been shown to inhibit TRAIL receptor-mediated apoptosis (Pan 1997b, Marsters 1997). Activity of endogenous levels of cell surface DcR1 has not been well characterized. DcR1 mRNA and cell surface expression in TECs is suppressed by the same conditions that induce apoptosis susceptibility. This suggests DcR1 suppression is a potential mechanism of apoptosis regulation in these cells. Although we cannot definitively prove that DcR1 is involved in TEC apoptosis regulation because its downregulation coincides with DR5 upregulation, it is on the cell surface and accessible to TRAIL binding. This could hypothetically reduce the amount of TRAIL available for binding to DR5 initiating the apoptotic signal (Ashkenazi 1999a).

Apoptosis plays an important role in autoimmune diseases (Rudin 1997, Elkon 1997, Mountz 1994, O'Reilly 1999). Death receptor pathways other than Fas and TNFR-1 have only recently become recognized as potentially important in disease pathogenesis (Wang 1999, Pai 1998). Specifically, inhibiton of TRAIL has been implicated in exacerbation of rheumatoid arthritis and experimental autoimmune encephalomyelitis in animal models (Song 2000, Hilliard 2001). The presence of TRAIL and TRAIL receptors in inflamed thyroids and regulation of the TRAIL apoptosis signaling pathway by the inflammatory cytokines TNFα, IL-1β and IFNγ, which are known to be present in autoimmune thyroids, suggests a role for these proteins in autoimmune thyroiditis (Bretz 2001). In addition, the presence of all these proteins in normal thyroids supports that this pathway is tightly regulated and is normally inhibited.

Inflammatory cytokines IFNγ, TNFα and IL-1β regulate TECs susceptibility to apoptosis by both Fas (Bretz 1999a) and TRAIL-mediated mechanisms, but this occurs only with specific and unique combinations of these cytokines. Interestingly, the conditions of maximum Fas-mediated apoptosis (IFNγ with either TNFα or IL-1β, and

all three combined) are different and exclusive of the conditions of maximum TRAIL-mediated apoptosis (TNFα with IL-1β). For example the addition of all three of these cytokines increases Fas-induced apoptosis but specifically inhibits TRAIL-induced apoptosis. This suggests that these complimentary activities have an important function and the cytokine milieu in the thyroid follicle microenvironment can determine the activity of apoptosis pathways. Moreover, the inhibition of the Fas and TRAIL apoptosis pathways occurs prior to their convergence to the common apoptotic pathway and involves distinct inhibitors that are differentially regulated.

In addition to TRAIL susceptibility, TRAIL expression is induced in TECs by certain combinations of cytokines. By mRNA analysis all three cytokines stimulate low levels of TRAIL expression with the greatest induction occurring under conditions of maximum susceptibility to Fas-mediated apoptosis (IFNγ combined with either TNFα and/or IL-1β) (Brety 1999a, Bretz 1999b). This suggests that TECs are becoming susceptible to FasL when they are simultaneously acquiring the capability of killing cells susceptible to TRAIL. Based on these observations we have proposed several models for the involvement of TRAIL in destructive thyroiditis (Bretz 2001). These are similar to models proposed for FasL (Giordano 1997, Dayan 1997): (1) Inflammatory cytokine sensitized TECs may be killed by autoimmune lymphocytes expressing TRAIL (2) TRAIL expressed by TECs may be involved in a defense mechanism for TECs to resist attack by killing autoimmune lymphocytes, or alternatively, (3) TRAIL expressed by TECs may result in self-destruction. Our results, the increased expression of TRAIL by TECs and the lack of TRAIL expression by lymphocytes in thyroiditis support the second hypothesis. Confirming this possibility, we have shown that CD3-positive lymphocytes isolated from an inflamed thyroid are susceptible to TRAIL-

induced apoptosis (unpublished observation). However, these models are not necessarily exclusive of each other. Future experiments will distinguish these scenarios. It has recently been reported that some but not all TRAIL preparations can kill normal human hepatocytes depending on the purification method and the presence of epitope tags (Jo 2000, Lawrence 2001). Our TRAIL was prepared by a method likely to produce hepatotoxicity (Chinnaiyan 2000). Regardless, the cytokine regulation of DR5 and DcR1, as well as the activity of the agonist DR5 antibody are independent from the TRAIL preparation. Based on this information it will be of interest to determine whether IFNγ treatment can protect normal human hepatocytes from TRAIL-mediated apoptosis in a manner similar to its rescue of TECs.

In summary, we have outlined mechanisms of TRAIL-mediated apoptosis in thyroid epithelial cells. This system of inducible and repressible TRAIL-mediated apoptosis will provide a useful experimental model for further studies about the regulation of TRAIL pathway. We have also demonstrated the presence of the involved proteins (TRAIL and its receptors) *in vivo*. This observation documents the relevance of these investigations to human disease states. Studies with animal models including transgenic mice as well as more in-depth studies in humans will be needed to clarify the role of these pathways in thyroid diseases.

5.2. Inflammatory cytokines transform nondestructive to destructive thyroiditis in EAT model

The hallmark of chronic thyroiditis in humans is the disruption of thyroid follicular structure with progressive glandular destruction leading to hypofunction (Vadutiu 1971, Weigle 1980). Immune mediated events underlie this process, and recent studies suggest that apoptosis mediates at least a portion of the follicular cell cytotoxicity

(Dremier 1994, Okayashu 1995). The reason that Hashimoto's disease is destructive while some other forms of human thyroiditis do not result in thyroid disruption is not known, but is an important issue. If thyroid destruction can be prevented in Hashimoto's disease, it would obviate the need for costly thyroid replacement therapy and the accompanying laboratory monitoring of thyroid-stimulating hormone concentrations. Understanding the pathogenic basis for Hashimoto's thyroiditis might also provide insights into other destructive autoimmune disorders such as type I diabetes and Addison's disease.

EAT is a well-defined murine model of thyroiditis induced by the injection of a homologous or heterologous thyroid antigen into genetically susceptible mice. EAT is characterized by the accumulation of lymphocytes in the thyroid gland of immunized animals and the presence of high titers of anti-Tg autoantibodies (Alimi 1998). In contrast to spontaneous thyroiditis in humans and animals, EAT is self-limited, resolving over several weeks, and does not result in follicular disruption (Alimi 1998). Our studies were able to reproduce traditional EAT by the injection of thyroid Ag, and this resulted in thyroid-infiltrating T lymphocytes. As expected, there was no significant evidence of increased apoptosis or follicular disruption in the thyroids from these animals. In contrast, the thyroids of mice immunized with thyroid Ag, but also systemically treated with two Th1 cytokines, IFNγ and TNFα, showed markedly sustained lymphocytic infiltration compared with control mice. These data indicated that the duration of thyroid infiltration was extended by cytokine treatment. It also coincided with enhanced apoptosis in the mouse thyroid with follicular disruption. In contrast, treatment with any single inflammatory cytokine did not result in enhanced lymphocytic infiltration or follicular disruption. Also, cytokine treatments that enhanced follicular disruption did not increase titers of antibodies to homologous or

heterologous Tg. Together, this suggests that thyroid disruption is enhanced in the presence of these cytokines without altering the overall immune response to thyroglobulin. These data also suggest that the humoral response alone is not adequate to mediate apoptotic destruction of the thyroid.

The role that IFNγ and TNFα play in modifying EAT and facilitating thyroid disruption is not definitive, but our studies suggest that target cell susceptibility to immune attack is altered by these molecules. Previous studies demonstrated that the combination of IFNγ and TNFα facilitates Fas-mediated apoptosis in primary human thyrocytes *in vitro* (Bretz 1999a), while a double dose of IFNγ or TNFα alone is not effective in mediating these effects (data not shown). A recent independent study also showed that Fas-induced apoptosis in HT was tightly regulated by Th1 cytokines (Stassi 2000). In the current studies, injection of anti-Fas antibody into the thyroids of mice yielded apoptosis only in animals pretreated with IFNγ and TNFα. This suggests that these cytokines may also facilitate Fas-mediated thyroid cell apoptosis in destructive thyroiditis in humans. In contrast, no study has shown that a single, inflammatory cytokine could induce destructive thyroiditis in EAT. The effects of IFNγ administered alone on the pathogenesis of autoimmune thyroiditis are still not clear. Experiments by Remy et al. (Remy 1987) induced EAT-like infiltrates, but not cytolysis through intrathyroidal injection of IFNγ, and the application of IFNγ-neutralizing Ab appeared to retard the development of EAT (Tang 1993). However, others have reported that the neutralization of endogenous IFNγ exacerbates granulomatous EAT (Stull 1992). IFNγ is also not required for the experimental induction of autoimmune thyroiditis, as traditional pathologic changes of EAT can occur in mice with disrupted IFNγ or IFN-rR genes (Alimi 1998). In contrast, synergy between IFNγ and TNFα has only been reported in the regulation of the Fas pathway (Baker 1998, Yasukawa 2000) and can

reduce Fas pathway inhibitors that are normally present in the thyroid (Arscott 1997). Thus, the induction of destructive thyroiditis in EAT is not simply the result of independent inflammatory actions of either IFNγ or TNFα, but relates to a unique interaction between these cytokines that facilitates apoptosis.

It is interesting that the addition of these two cytokines is necessary to mediate destructive thyroiditis in EAT, especially since the immune cells infiltrating the thyroid in this model are Th1 and should produce both of these cytokines. CFA generates a Th1 response (Grun 1989), so one might expect the presence of these cytokines in immunized animals. However, it is possible that the Th1 environment in the mouse thyroid did not produce adequate amounts of both cytokines to reach a threshold to efficiently activate the Fas apoptotic pathway(s). This could be because the primary inflammatory response in EAT is at the site of immunization and not in the thyroid as it is in spontaneous thyroiditis. However, it may be that immunogenetic factors that control the production of Th1 cytokines are not adequate to produce these cytokines in the thyroids of some mice and potentially of humans.

Cytotoxic lymphocytes kill their target cells predominantly through granule exocytosis with perforin/granzyme and/or the Fas-FasL system (Kagi 1996, Henkart 1994), with the Fas-FasL pathway operative in both $CD4^+$ and $CD8^+$ T cells (Stassi 2000, Ju 1994, Ju 1995, Stalder 1994). Although granule exocytosis-mediated cytotoxicity against thyroid follicular cells might play a role in the destruction of HT thyrocytes (Wan 1998), increasing evidence suggests that the Fas-FasL pathway is central to this process (Stalder 1994). Because FasL is present on activated T lymphocytes (Suda 1995, Bossi 1999) and the lymphocytic infiltration of the thyroid of IFNγ- and TNFα-treated EAT includes both $CD4^+$ and $CD8^+$ T effector cells (Stassi 2000), Fas on the surface of thyrocytes should mediate thyroid apoptosis. However, prior studies indicated that this

pathway is normally blocked in thyroid cells (Arscott 1997, Bretz 1999a). This was confirmed in our animals by the inability of an agonist anti-Fas Ab to induce apoptosis when injected into the thyroid gland. This is reversed by treatment with IFNγ and TNFα, but it is unclear what mechanism is used by the two cytokines to influence the Fas pathway. A previous study demonstrated that susceptibility to Fas-induced death by the combination of IFNγ and TNFα in thyroid cells correlated with an increase in expression of a tunicamycin-inhibitable high molecular weight form of Fas (Bretz 1999a). This suggested that Fas glycosylation might play an important role in regulating Fas signaling. Thus, it appears that altering the environment in the thyroid to activate the Fas pathway is adequate to allow the induction of apoptosis and thyroid disruption in EAT.

In summary, the combination of IFNγ and TNFα enhanced thyrocyte apoptosis through the Fas pathway and induced follicular disruption in EAT. This may help to explain the differences between EAT and HT, because the thyroid in patients with HT has a chronic inflammatory environment enriched in Th1 cytokines such as IFNγ and TNFα and increased thyrocyte apoptosis (Stassi 2000). These results also suggest that other disorders with Th1 immune response may result in cellular apoptosis through the Fas-FasL pathway due to the cytokine environment. Genetic differences in the cytokine regulation of this pathway involving apoptosis regulatory proteins may predispose individuals to these disorders.

5.3. Aberrant regulation of apoptosis in thyroid cells from goiter nodules

A proper balance between cell proliferation and cell death is required to maintain tissue homeostasis. Goitrogenesis is a very slow process, unlike cancer, which is caused by

increased cell proliferation. It is possible that a decrease in cell death can contribute to the accumulation of cells in goiter nodules. Normal thyroid cells are resistant to all known death ligands, despite the constitutive expression of their respective death receptors (Arscott 1997, Bretz 1999a, Bretz 1999a, Bretz and Mezosi 2002), but can be sensitized to death induction by these ligands using proinflammatory cytokines (Bretz 1999a, Bretz and Mezosi 2002). In contrast to normal cells, the majority of goiter cell populations are not sensitized to TRAIL- or Fas-mediated apoptosis by cytokine pretreatment. This suggests a functional decrease in death receptor-mediated apoptotic activity in goiter-derived primary thyroid cells and indicates that there is an altered regulation of these death pathways in goiter cells. Moreover, the sensitivity to TRAIL-induced cell death inversely correlated with the goiter size. This raises the possibility that TRAIL is important in maintaining normal thyroid cell populations and TRAIL resistance contributes to the development of nodular goiter.

The death receptor pathways are controlled by a variety of mechanisms, from the expression of death and decoy receptors to the control of intracellular signaling. In various cell types, the resistance to TRAIL has been reported to be mediated by distinct mechanisms (Zhang 2000a). The lack of surface death receptor expression was decisive in the resistance of melanocytes to TRAIL, while expression of a decoy receptor (DcR1) was reported to be important in the protection of endothelial cells (Zhang 2000a). Fibroblasts were protected from TRAIL-induced apoptosis by intracellular inhibitors (Zhang 2000a). In our studies, TRAIL resistance in untreated normal thyroid cells is likely due to the low level of death receptor surface expression (Bretz and Mezosi 2002). However, the presence of intracellular inhibitors to mediate TRAIL resistance can not be ruled out, because the inhibition of protein synthesis makes normal

thyroid cells susceptible to TRAIL, possibly through selective loss of labile inhibitors (Bretz 1999b).

In TNFα/IL-1β pretreated normal thyrocytes, TRAIL signal is transmitted by DR5 (Bretz and Mezosi 2002). In these cells, the treatment with TNFα/IL-1β upregulated overall levels of DR5 protein and cell surface expression, providing a possible mechanism for TRAIL sensitization. In TRAIL resistant goiter cells, there was no change in total DR5 protein expression after TNFα/IL-1β treatment but DR5 surface protein expression was increased similar to normal cells. Despite having cell surface receptor, DR5 signal transmission was not accomplished in these cells as confirmed in studies using an agonist anti-DR5 antibody. Thus, in goiter cells that did not respond to TNFα/IL-1β, intracellular mechanisms appeared to play a role in TRAIL resistance. A screening of well-known intracellular inhibitors (cFLIP, bcl-2, IAPs) did not identify the source for goiter cell resistance, raising the possibility that other inhibitors or a downregulation of proapoptotic proteins may be responsible.

One potential mechanism behind the downregulation of proapoptotic proteins in resistant goiters could be an increase in protein degradation. Most intracellular proteins are degraded by the proteasome complex. In a subset of goiters increased proteasome activity was found, which was correlated with the resistance of goiter cells to both death ligands. Low doses of proteasome inhibitors sensitized the resistant goiters to TRAIL, supporting a causal relationship between elevated proteasome activity and apoptosis resistance. The essential role of the proteasome in cell survival is well known and many studies showed that proteasome inhibitors can induce apoptosis or sensitize cells to TRAIL and FasL (Drexler 1997, Orlowski 1999, Cui 1997, Van Valen 2000, Kim 2001). However, this is the first study of proteasome activity in normal and goiter-

derived thyroid cells and the first demonstration of a positive correlation between the proteasome activity and resistance to death receptor-mediated apoptosis.

Primary cell cultures provide a unique opportunity to investigate the normal regulation of death receptor-mediated apoptosis and altered regulation in specific disease conditions. Concerns might be raised that the *in vitro* culture of these cells might alter their resistance to apoptosis. This is controlled since normal and goiter cells were cultured simultaneously for similar time periods and under identical conditions. However, there might be differences in serum-dependent signaling between normal and nodular tissue. The relationship of growth promoting signaling and resistance to apoptosis should be addressed in future examinations. These *in vitro* results need to be confirmed in forthcoming *in vivo* studies.

In summary, while normal thyroid cells *in vitro* can be sensitized to TRAIL and FasL by inflammatory cytokines, the majority of goiter samples show resistance to death ligands after cytokine pretreatment. The resistance of goiter cells to TRAIL is not due to the altered surface expression of death receptors or the overexpression of cFLIP, bcl-2 and IAPs. The increased proteasome activity in a subset of resistant goiters and the ability of proteasome inhibitors to sensitize resistant cells to TRAIL suggest that the proteasome is an important regulator of apoptosis resistance. Moreover, the inverse correlation of goiter size with the sensitivity to TRAIL-mediated apoptosis suggests a relationship to goiter pathogenesis.

5.4. SCID mouse model for the investigation of human thyroid cancers

Beside autoimmune disorders, another major implication of apoptosis studies is to treat human cancers. TRAIL was reported as a promising anti-cancer agent that selectively kills tumor cells (Ashkenazi 1999, Bonavida 1999). Thyroid carcinoma cell lines

resistant to FasL were found to be sensitive to TRAIL (Mitsiades 2000). However, cancer cell lines are not optimal targets to study the death receptor-mediated apoptosis, because their responses to death ligands are markedly different from primary tumor cells. In order to overcome the limitations of earlier animal models and *in vitro* experiments, we used the hu-PBL SCID mouse model for the investigation of human thyroid tumors. Previously, different types of human cancers were successfully transplanted into the SCID mice (Mueller 1991, Williams 1993, Hendrickson 1993, Juhasz 1993, Paine-Murietta 1997) and promising initial results of cancer immunotherapy were recently reported using these animals (Tam 1999, Bonnet 1999, Hu 1999, Schultes 1999, Bauer 1999, Cochlovius 1999). Autoimmune thyroid diseases (AITD) were also extensively studied, but as a model of AITD the hu-PBL-SCID mouse has not proved to be an ideal system (Volpe 1996). The homing of PBMCs to Graves' and Hashimoto's thyroid tissues was demonstrated (Resetkova 1995), destruction of thyroid follicles and thyroid autoantibody production was detected (Volpe 1993) but the human IgG production was variable and declined after the sixth week (Volpe 1996). Others have not found any thyroid follicular destruction, the lymphocytic infiltration decreased over time and the HLA-DR expression disappeared from the thyrocytes (Martin 1992, Martin 1994). Another problem was the unintentional reconstitution of the human immune system by intrathyroidal lymphocytes (without engrafted PBMCs), which required the use of nude mice to kill passenger lymphocytes and normalize thyroid tissue from autoimmune thyroid diseases before engraftment to SCID mice (Volpe 1993). Consequently, the reproduction of AITDs in their complexity was unsuccessful in SCID mice (Martin 1994, Volpe 1996).

The clinical and immunological aspects of differentiated thyroid cancers strongly suggest a crucial role for the immune system in the control of this disease, and support

the potential effectiveness of immunotherapy (Baker 1993, Baker 1995, Boyd 1996). Patients with thyroid cancer often have antithyroid antibodies, and cytotoxic immune activity has been reported in TILs isolated from thyroid cancer (Juhasz 1989, Bagnasco 1989). In addition to T cell mediated immunity, the natural immunity also participates in anti-tumor activity (Baker 1993), an increased prevalence of natural killer-like and lymphokine-activated killer activity in TILs of papillary carcinomas was reported (Bagnasco 1994). In contrast, thyrocyte specific killer cell activity has been found to be decreased in the serum of patients with thyroid carcinoma, but NK activity was normal against a standard tumor cell target (Sack 1987). NK cells may have different subpopulations and the exact mechanism of local immunity is poorly understood. Another important possibility is the killing of thyroid neoplastic cells by macrophages, which was recently published (Fiumara 1997).

In our experiments, all types of malignant thyroid tumors and a benign follicular adenoma were investigated to determine the growth rate of different histological types and to check the metastatic features of thyroid tumors in SCID mice. The engraftment of tumors was successful in every case. The growth of xenotransplants was dependent on the histological type. The growth rate of DTCs was quite slow. No distant metastasis was observed. Anaplastic cancer grafts were locally invasive, otherwise the organs of mice were not infiltrated with cancer cells. The histological characters of the tumors growing in the animals proved to be unchanged, except one case of papillary cancer, where an anaplastic cancer developed in one mouse while the original papillary structure remained in the other mouse. The presence of a small, unrecognized anaplastic focus is the most probable explanation of this observation. The most interesting outcome was the gradual decrease and disappearance of one papillary graft in the presence of human lymphocytes. The donor was the only patient who had diffuse

lymphocytic thyroiditis with germinative centres, her papillary cancer was small, without any lymph node metastasis. The slower growth and the presence of lymphocytes (probably repopulating) 16 weeks after transplantation in another case reconstituted with human PBLs suggests the sensitization of the immune system against thyroid cancer in this patient.

Regarding the reconstitution of the human immune system, our results were similar to the observations received by lymphocyte transfer from humans with autoimmune thyroid diseases (Martin 1992, Volpe 1993, Martin 1994, Volpe 1996). From one hand the engraftment of PBLs did not result in an entirely functional immune system in mice, from the other hand the lymphocytic infiltration in tumors was able to unintentionally reconstitute a certain level of human immune system. The human immunoglobulin levels showed immune reconstitution in both groups of mice, with or without the engraftment of human PBLs, showing that lymphocytes in the tumor grafts were sufficient to achieve comparable level of immune reconstitution. IgG production decreased over time (data not shown). This finding has two important consequences. First, TILs interfere with the results when one would like to examine thyroid cancer tissue without lymphocytes. Second, the large number of TILs may provide a unique possibility to detect the subpopulations and function of these cells. More than 15% of TILs from papillary cancer tissue was found to coexpress CD4 and CD8 antigen (Lee 1996). Thyroid derived TILs displayed an intense cytolytic activity against NK-sensitive tumor cells and in more than 50% of cases against autologous tumor cells *in vitro* (Lee 1996). Human papillary cancer xenografts with TILs were found to produce thyroglobulin antibody but not thyroperoxidase-antibody (Kawai 1998). No studies on death ligand expression of TILs and the involvement of apoptosis in thyroid tumor cell

killing were reported. Our *in vivo* observations may open the way for further elucidation of lymphocyte-DTC interactions.

The expression of HLA-DR antigens in DTC was investigated in detail but its role in immune recognition and anti-tumor immunity was not clarified (Feinmesser 1996, Goldsmith 1988). Recently, it was suggested that HLA-DR expression in the absence of lymphocytic infiltration might be the marker of immune tolerance and indicated poor prognosis (Matsubayashi 1995). In another study HLA-DR expression of papillary cancer cells was found to be strongly constitutive, but it was also affected by TILs (Kawai 1998). In our investigation HLA-DR expression was found to be constitutive in 2 cases and maintained by infiltrating lymphocytes in 3 cases. Examination of more cases seems to be necessary to clarify the importance of HLA-DR expression of papillary cancer.

In summary, it has been shown for the first time that papillary, follicular, anaplastic and medullary cancer can successfully be grown in SCID mice. Growth rate, as expected, was more rapid in undifferentiated cancers. The hu-PBL-SCID mouse model is useful for the investigation of biological characteristics of human thyroid cancers and seems to be suitable for the examination of anti-tumor immunity of patients. However, further improvement of the immune reconstitution technique is warranted to evaluate novel therapeutic approaches to thyroid cancer.

6. SUMMARY

Death receptor mediated apoptosis plays an important role in a number of thyroid disorders. The present study demonstrated for the first time the *in vivo* presence of TRAIL and its receptors in the normal thyroid gland and in Hashimoto's thyroiditis. The TRAIL-mediated apoptosis of thyroid epithelial cells is regulated by inflammatory cytokines. IL-1β alone and in combination with TNFα induces sensitivity to TRAIL, while the further addition of IFNγ makes these cells resistant to apoptosis induced by TRAIL. This pattern of TRAIL sensitivity correlates with levels of cell surface expression of TRAIL receptors. IL-1β/TNFα enhancement of DR5 surface expression provides a mechanism for TRAIL sensitivity. We have further documented that the signal is mediated through DR5 by the use of DR5 specific agonist antibody.

Confirming the essential role of proinflammatory cytokines in the regulation of apoptosis *in vivo*, the combination of IFNγ and TNFα enhanced thyrocyte apoptosis through the Fas pathway and transformed nondestructive to destructive thyroiditis in a murine model of experimental autoimmune thyroiditis.

The majority of goiter-derived thyroid cells cannot be sensitized to TRAIL and/or Fas-mediated apoptosis by inflammatory cytokines, which represents a new aspect of aberrant growth regulation in goiter nodules. The increased proteasome activity associated with this resistance suggests that the proteasome may be an important regulator of apoptosis in nodular goiter.

Finally, the hu-PBL-SCID mouse was established for the *in vivo* investigation of human thyroid cancer. This model is appropriate to examine the biological characteristics of thyroid cancers but warrants improvement to analyze the interaction between the immune system and tumor cells.

7. REFERENCES

Ajjan RA, Watson PF, Weetman AP. 1996. Cytokines and thyroid function. Adv. Neuroimmunol. 6:359-386.

Alimi E, Huang S, Brazillet MP, Charreire J. 1998. Experimental autoimmune thyroiditis (EAT) in mice lacking the IFN-γ receptor gene. Eur. J. Immunol. 28:201-208.

Amino N, Pysher T, Cohen EP, DeGroot LJ. 1975. Immunologic aspects of human thyroid cancer. Humoral and cell-mediated immunity and a trial of immunotherapy. Cancer 36:963-973.

Aoki N, DeGroot J. 1979. Lymphocyte blastogenic response to human thyroglobulin in Graves' disease, Hashimoto's thyroiditis, and metastatic thyroid cancer. Clin. Exp. Immunol. 38:523-530.

Arends MJ, Wyllie AH. 1991. Apoptosis: mechanisms and roles in pathology. Int. Rev. Exp. Pathol. 32:223-254.

Arscott PL, Baker JR, Jr. 1998. Apoptosis and thyroiditis. Clin. Immunol. Immunopathol. 87:207-217.

Arscott PL, Knapp J, Rymaszewski M, Bartron JL, Bretz JD, Thompson NW, Baker JR, Jr. 1997. Fas (APO-1, CD95)-mediated apoptosis in thyroid cells is regulated by a labile protein inhibitor. Endocrinology 138:5019-5027.

Ashkenazi A, Dixit VM. 1999a. Apoptosis control by death and decoy receptors. Curr. Opin. Cell. Biol. 11:255-260.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge AL, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH. 1999b. Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. 104:155-62.

Asmis LM, Kaempf J, Gruenigen CV, Kimura ET, Wagner HE, Studer H. 1996. Aquired and naturally occurring resistance of thyroid follicular cells to the growth inhibitory action of transforming growth factor-β1 (TGF-β1). J. Endocrinol. 149:485-496.

Bagnasco M, Venuti D, Paolieri F, Torre G, Ferrini S, Canonica GW. 1989. Phenotypic and functional analysis at the clonal level of infiltrating T lymphocytes in papillary carcinoma of the thyroid: Prevalence of cytolytic T cells with natural killer-like or lymphokine-activated killer activity. J. Clin. Endocrinol. Metab. 69:832-836.

Baker JR, Jr., Fosso CK. 1993. Immunological aspects of cancers arising from thyroid follicular cells. Endocr. Rev. 14:729-746.

Baker JR. Jr. 1995. The immune response to papillary thyroid cancer. J. Clin. Endocrinol. Metab. 80:3419-3420.

Baker SJ, Reddy EP. 1998. Modulation of life and death by the TNF receptor superfamily. Oncogene 17:3261-3270.

Baker JR, Jr. 1999. Editorial: Dying (Apoptosing?) for a consensus on the Fas death pathway in the thyroid. J. Clin. Endocrinol. Metab. 84:2593-2595.

Batteux F, Tourneur L, Trebeden H, Charreire J, Chiocchia G. 1999 Gene therapy of experimental autoimmune thyroiditis by in vivo administration of plasmid DNA coding for Fas ligand. J. Immunol. 162: 603-608.

Bauer S, Renner C, Juwana JP, Held G, Ohnesorge S, Gerlach K and Pfreundschuh M. 1999. Immunotherapy of human tumors with T-cell-activating bispecific antibodies: stimulation of cytotoxic pathways in vivo. Cancer. Res. 59:1961-1965.

Benoist C, Mathis D. 1997. Cell death mediators in autoimmune diabetes--no shortage of suspects. Cell 89:1-3.

Bidey SP, Hill DJ, Eggo MC. 1999. Growth factors and goitrogenesis. J. Endocrinol. 160:321-332.

Bisi H, Fernades VSO, De Camargo RYA, Koch L, Abdo AH, De Brito T. 1989. The prevalence of unsuspected thyroid pathology in 300 sequential autopsies with special reference to the incidental carcinoma. Cancer 64:1888-1893.

Bonavida B, Ng CP, Jazirehi A, Schiller G, Mizutani Y. 1999. Selectivity of TRAIL-mediated apoptosis of cancer cells and synergy with drugs: the trail to non-toxic cancer therapeutics. Int. J. Oncol. 15:793-802.

Bonnet D, Warren EH, Greenberg PD, Dick JE, Riddell SR. 1999. CD8(+) minor histocompatibility antigen-specific cytotoxic T lymphocyte clones eliminate human acute myeloid leukemia stem cells. Proc. Natl. Acad. Sci. USA 96:8639-8644.

Borgerson KL, Bretz JD, Baker JR, Jr. 1999. The role of Fas-mediated apoptosis in thyroid autoimmune disease. Autoimmunity 30:251-264.

Bosma GC, Custer RP, Bosma MJ. 1983. A severe combined immunodeficiency mutation in the mouse. Nature 301:527-530.

Bossi G, Griffiths GM. 1999. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. Nature Med. 5:90-96.

Boyd CM, Baker JR, Jr. 1996. The immunology of thyroid cancer. Endocrinol. Metab. Clin. North. Am. 25:159-179.

Braley-Mullen, H., Sharp GC, Tang H, Chen K, Kyriakos M, Bickel JT. 1998. Interleukin-12 promotes activation of effector cells that induce a severe destructive granulomatous form of murine experimental autoimmune thyroiditis. Am. J. Pathol. 152:1347-1358.

Bretz JD, Baker JR, Jr. 2001. Apoptosis and autoimmune thyroid disease: following a TRAIL to thyroid destruction? Clin. Endocrinol. 55:1-11.

Bretz JD, Arscott PL, Myc A, Baker JR, Jr. 1999. Inflammatory cytokine regulation of Fas-mediated apoptosis in thyroid follicular cells. J. Biol. Chem. 274:25433-25438.

Bretz JD, Mezosi E, Giordano TJ, Gauger PG, Thompson NW, Baker JR, Jr. 2002. Inflammatory cytokine regulation of TRAIL-mediated apoptosis in thyroid epithelial cells. Cell Death Diff. 9:274-286.

Bretz JD, Rymaszewski M, Arscott PL, Myc A, Ain KB, Thompson NW, Baker JR, Jr. 1999. TRAIL death pathway expression and induction in thyroid follicular cells. J. Biol. Chem. 274:23627-23632.

Charreire J. 1989. Immune mechanisms in autoimmune thyroiditis. Adv. Immunol. 46:263-334.

Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway CA, Jr., Matis LA. 1997. The role of Fas in autoimmune diabetes. Cell 89:17-24.

Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD, Rehemtulla A. 2000. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc. Natl. Acad. Sci. USA 97:1754-1759.

Cochlovius B, Perschl A, Adema GJ, Zoller M. 1999. Human melanoma therapy in the SCID mouse: in vivo targeting and reactivation of melanoma specific cytotoxic T cells by bi-specific antibody fragments. Int. J. Cancer. 81:486-493.

Cui H, Matsui K, Omura S, Schauer SR, Matulka RA, Sonenshein GE. 1997. Proteasome regulation of activation-induced T cell death. Proc. Natl. Acad. Sci. USA 94:7515-7520.

Dayan CM, Elsegood KA, Maile R. 1997. FasL expression on epithelial cells: the Bottazzo-Feldman hypothesis revisited. Immunol. Today 18:203.

Del Prete GF, Tiri A, De Carli M, Mariotti S, Pinchera A, Chretien I, Romagnani S, M. Ricci. 1989. High potential to tumor necrosis factor α(TNF-α) production of thyroid infiltrating T lymphocytes in Hashimoto's thyroiditis: a peculiar feature of destructive thyroid autoimmunity. Autoimmunity 4:267-276.

Derwahl M. 1996a. Editorial: TSH receptor and Gs-α gene mutations in the pathogenesis of toxic thyroid adenomas – A note of caution. J. Clin. Endocrinol. Metab. 81:2783-2785.

Derwahl M. 1996b. Molecular aspects of the pathogenesis of nodular goiters, thyroid nodules and adenomas. Exp. Clin. Endocrinol. Diabetes 104 Suppl 4:32-35.

Dremier S, Golstein J, Mosselmans R, Dumont JE, Galand P, Robaye B. 1994. Apoptosis in dog thyroid cells. Biochem. Biophys. Res. Commun. 200:52-58.

Drexler HCA. 1997. Activation of cell death program by inhibition of proteasome function. Proc. Natl. Acad. Sci. USA 94:855-860.

Elkon KB. 1997. Apoptosis and autoimmunity. J. Rheumat. 24:6-7.

Feinmesser M, Stern S, Mechlis-Frish S, Lubin E, Feinmesser R, Kristt D. 1996. HLA-DR expression and lymphocytic infiltration in metastatic and non-metastatic papillary carcinoma of the thyroid. Eur. J. Surg. Oncol. 22:494-501.

Fiumara A, Belfiore A, Russo G, Salomone E, Santonocito GM, Ippolito O, Vigneri R, Gangemi P. 1997. In situ evidence of neoplastic cell phagocytosis by macrophages in papillary thyroid cancer. J. Clin. Endocrinol. Metab. 82:1615-1620.

Foulis AK. 1996. The pathology of the endocrine pancreas in type 1 (insulin-dependent) diabetes mellitus. APMIS 104:161-167.

Frank S, Kohler U, Schackert G, Schackert HK. 1999. Expression of TRAIL and its receptors in human brain tumors. Biochem. Biophys. Res. Comm. 257:454-459.

French LE, Tschopp J. 1997. Thyroiditis and hepatitis: Fas on the road to disease. Nature Med. 3:387-388.

Frohman MJ, Francfort W, Cowing C. 1991. T-dependent destruction of thyroid isografts exposed to IFN-γ. J. Immunol. 146:2227-2234.

Gartner R, Veitenhansl M, Aktas J, Schopohl D. 1996. Role of basic fibroblast growth factor in the pathogenesis of nodular goiter. Exp. Clin. Endocrinol. Diabetes 104 Suppl 4:36-38.

Giordano C, Stassi G, De Maria R, Todaro M, Richiusa P, Papoff G, Ruberti G, Bagnasco M, Testi R, Galluzzo A. 1997. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science 275:960-963.

Giordano C, Richiusa P, Sbriglia MS, Pizzolanti G. 1998. The role of FAS/FASLig and apoptosis in beta-cell death in animal models: relevance for human IDDM. Diabetes-Metabolism Reviews 14:194-195.

Goldsmith NK, Dikman S, Bermas B, Davies TF, Roman S. 1988. HLA class II antigen expression and the autoimmune thyroid response in patients with benign and malignant thyroid tumors. Clin. Immunol. Immunopathol. 48:161-173.

Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. Science.270:1189-1192.

Griffith TS, Lynch DH. 1998a. TRAIL: a molecule with multiple receptors and control mechanisms. Curr. Opin. Immunol. 10:559-563.

Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. 1998b. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J. Immunol. 161:2833-2840.

Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH, Smith CA, Goodwin RG, Kubin MZ. 1999. Functional analysis of TRAIL receptors using monoclonal antibodies. J. Immunol. 162:2597-2605.

Grun JM, Mauter PH. 1989. Different T helper subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin-1 in proliferative responses. Cell. Immunol. 121:134-137.

Gura T. 1997. How TRAIL kills cancer cells, but not normal cells. Science 277:768.

Hamilton F, Black M, Farquharson MA, Stewart C, Foulis AK. 1991. Spatial correlation between thyroid epithelial cells expressing class II MHC molecules and interferon-γ-containing lymphocytes in human thyroid autoimmune disease. Clin. Exp. Immunol. 83:64-68.

Hammond LJ, Lowdell MW, Cerrano PG, Goode AW, Bottazzo GF, Mirakian R. 1997. Analysis of apoptosis in relation to tissue destruction associated with Hashimoto's autoimmune thyroiditis. J. Pathol. 182:138-144.

Hendrickson EA. 1993. The SCID mouse: Relevance as an animal model system for studying human disease. Am. J. Path. 143:1511-1522.

Henkart PA, 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity 1:343-346.

Hilliard B, Wilmen A, Seidel C, Liu TS, Goke R, Chen Y. 2001. Roles of TNF-Related Apoptosis-Inducing Ligand in Experimental Autoimmune Encephalomyelitis. J. Immunol. 166:1314-1319.

Hu Z, Sun Y, Garen A. 1999. Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. Proc. Natl. Acad. Sci. USA. 96:8161-8166.

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima SI, Sameshima M, Hase A, Seto Y, Nagata S. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66:233-243.

Iwanuma Y, Chen FA, Egilmez NK, Takita H, Bankert RB. 1997. Antitumor immune response of human peripheral blood lymphocytes coengrafted with tumor into severe combined immunodeficient mice. Cancer Res. 57:2937-2942.

Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, Strom SC. 2000. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nature Med. 6:564-567.

Ju ST, Cui H, Panka DJ, Ettinger R, Marshak-Rothstein A. 1994. Participation of target Fas protein in apoptosis pathway induced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. Proc. Natl. Acad. Sci. USA 91:4185-4189.

Ju ST, Panka DJ, H. Cui H, Ettinger R, el-Khatib M, Sherr DH, Stanger BZ, Marshak-Rothstein A. 1995. Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 373:444-448.

Juhasz F, Boros P, Szegedi G, Balazs G, Suranyi P, Kraszits E, Stenszky V, Farid NR. 1989. Immunogenetic and immunologic studies of differentiated thyroid cancer. Cancer 63:1318-1326.

Juhasz I, Albelda SM, Elder DE, Murphy GF, Adachi K, Herlyn D, Valyi-Nagy IT, Herlyn M. 1993. Growth and invasion of human melanomas in human skin grafted to immunodeficient mice. Am. J. Pathol. 143:528-537.

Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Ann. Rev. Immunol. 14:207-232.

Kamma H, Fujii K, Ogata T. 1988. Lymphocytic infiltration in juvenile thyroid carcinoma. Cancer 62:1988-1993.

Kashima K, Yokoyama S, Noguchi S, Murakami N, Yamashita H, Watanabe S, Uchino S, Toda M, Sasaki A, Daa T, Nakayama I. 1998. Chronic thyroiditis as a favorable prognostic factor in papillary thyroid carcinoma. Thyroid 8:197-202.

Kawai K, Resetkova E, Enomoto T, Fornasier V, Volpé R. 1998. Is human leukocyte antigen-Dr and intercellular adhesion molecule-1 expression on human thyrocytes consitutive in papillary thyroid cancer? Comparative studies of human thyroid xenografts in severe combined immunodeficient and nude mice. J. Clin. Endocrinol. Metab. 83:157-164.

Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Kawabe Y, Ishikawa N, Ito K, Nagataki S. 1996. Thyroid-stimulating hormone inhibits Fas antigen-mediated apoptosis of human thyrocytes *in vitro*. Endocrinology. 137:3163-3169.

Kawakami Y, Kuzuya N, Watanabe T, Uchiyama Y, Yamashita K. 1990. Induction of experimental thyroiditis in mice by recombinant interferon γ administration. Acta Endocrinol. 122:41-48.

Kikuchi S, Hiraide H, Tamakuma S, Yamamoto M. 1997. Expression of wild-type p53 tumor suppressor gene and its possible involvement in the apoptosis of thyroid tumors. Surg. Today 27:226-233.

Killinger WA Jr., Dorofi DB, Tinsley EA Jr., Keagy BA, Johnson G Jr. 1992. Flow cytometric analysis of organ preservation-induced endothelial cell membrane damage. Ann. Thorac. Surg. 53:472-476.

Kim K. 2001. Proteasome inhibitors sensitize human vascular smooth muscle cells to Fas (CD95)-mediated death. Biochem. Biophys. Res. Comm. 281:305-310.

Kim K, Fisher MJ, Xu SQ, el-Deiry WS. 2000. Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin. Cancer Res. 6:335-346.

Kotani T, Aratake Y, Hirai K, Fukazawa Y, Sato H, Ohtaki S. 1995. Apoptosis in thyroid tissue from patients with Hashimoto's thyroiditis. Autoimmunity 20:231-236.

Kothny-Wilkes G,D, Kulms D, Poppelmann B, Luger TA, Kubin M, Schwarz T. 1998. Interleukin-1 protects transformed keratinocytes from tumor necrosis factor-related apoptosis-inducing ligand. J. Biol. Chem. 273:29247-29253.

Kripke ML. 1988. Immunoregulation of carcinogenesis: past, present, and future. J. Natal. Cancer. Inst. 80:722-727.

Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, Hooley J, Sherwood S, Pai R, Leung S, Khan L, Gliniak B, Bussiere J, Smith CA, Strom SS, Kelly S, Fox JA, Thomas D, Ashkenazi A. 2001. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nature Med. 7:383-385.

Lee RS, Schlumberger M, Caillou B, Pages F, Fridman WH, Tartour E. 1996. Phenotypic and functional characterisation of tumor-infiltrating lymphocytes derived from thyroid tumors. Eur. J. Cancer. 32A:1233-1239.

Leverkus M, Neumann M, Mengling T, Rauch CT, Brocker EB, Krammer PH, Walczak H. 2000. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res. 60:553-559.

Lin KI, Baraban JM, Ratan RR. 1998. Inhibition versus induction of apoptosis by proteasome inhibitors depends on concentration. Cell Death Differ. 5:577-583.

Lowin B, Hahne M, Mattmann C, Tschopp J. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature 370:650-652.

Luttmann W, Opfer A, Dauer E, Foerster M, Matthys H, Eibel H, Schulze-Osthoff K, Kroegel C, Virchow JC. 1998. Differential regulation of CD95 (Fas/APO-1) expression in human blood eosinophils. Eur. J. Immunol. 28:2057-65.

Maiorano E, Ambrosi A, Giorgino R, Fersini M, Pollice L, Ciampolillo A. 1994. Insulin-like Growth Factor 1 (IGF-1) in multinodular goiters: A possible pathogenetic factor. Pathol. Res. Pract. 190:1012-1016.

Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P, Ashkenazi A. 1997. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr. Biol. 7:1003-1006.

Martin A, Kimura H, Thung S, Fong P, Shultz LD. Davies TF. 1992. Characteristics of long-term human thyroid peroxidase autoantibody secretion in scid mice transplanted

with lymphocytes from patients with autoimmune thyroiditis. Int. Arch. Allergy. Immunol. 98:317-323.

Martin A, Valentine M, Unger P, Yeung SW, Shultz LD. Davies TF. 1994. Engraftment of human lymphocytes and thyroid tissue into scid and rag2-deficient mice: antibodiesent progression of lymphocytic infiltration. J. Clin. Endocrinol. Metab. 79:716-723.

Matsubayashi S, Kawai K, Matsumoto Y, Mukuta T, Morita T, Hirai K, Matsuzuka F, Kakudoh K, Kuma K, Tamai H. 1995. The correlation between papillary thyroid carcinoma and lymphocytic infiltration in the thyroid gland. J. Clin. Endocrinol. Metab. 80:3421-3423.

Mitsiades N, Poulaki V, Mitsiades C, Tsokos M. 2001. Ewing's sarcoma family tumors are sensitive to Tumor Necrosis Factor-related Apoptosis-inducing Ligand and express Death Receptor 4 and Death Receptor 5. Cancer Res. 61:2704-2712.

Mitsiades N, Poulaki V, Tseleni-Balafouta S, Koutras DA, Stamenkovic I. 2000. Thyroid carcinoma cells are resistant to fas-mediated apoptosis but sensitive to tumor necrosis factor-related apoptosis-inducing ligand. Cancer Res. 60:4122-4129.

Moore D, Ohene-Fianko D, Garcia B, Chakrabarti S. 1998. Apoptosis in thyroid neoplasms: relationship with p53 and bcl-2 expression. Histopathology 32:35-42.

Mosier DE, Gulizia RJ, Baird SM, Wilson DB. 1988. Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature 335:256-259.

Mountz JD, Wu J, Cheng J, Zhou T. 1994. Autoimmune disease. A problem of defective apoptosis. Arthritis & Rheumatism 37:1415-1420.

Mountz JD, Zhang HG, Hsu HC, Fleck M, Wu J, Al-Maini MH, Zhou T. 1999. Apoptosis and cell death in the endocrine system. Recent Progress in Hormone Research 54:235-68.

Mountz JD, Zhang HG, Hsu HC, Fleck M, Wu J, al-Maini MH, Zhou T. 1999. Apoptosis and cell death in the endocrine system. Recent Progress in Hormone Research 54:235-68; discussion 269.

Mueller BM, Reisfeld RA. 1991 Potential of the scid mouse as a host for human tumors. Cancer Met. Rev. 10:193-200.

Nagata S. 1996. Fas-induced apoptosis, and diseases caused by its abnormality. Genes to Cells. 1:873-879.

Ohsako S, Elkon KB. 1999. Apoptosis in the effector phase of autoimmune diabetes, multiple sclerosis and thyroiditis. Cell Death Differ. 6:13-21.

Okayasu I, Saegusa M, Fujiwara M, Hara Y, Rose NR. 1995. Enhanced cellular proliferative activity and cell death in chronic thyroiditis and thyroid papillary carcinoma. J. Cancer Res. Clin. Oncol. 121:746-752.

O'Reilly LA, Strasser A. 1999. Apoptosis and autoimmune disease. Inflammation Res. 48:5-21.

Orlowski RZ. 1999. The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ. 6:303-313.

Pai SI, Wu GS, Ozoren N, Wu L, Jen J, Sidransky D, El-Deiry WS. 1998. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. Cancer Res. 58:3513-3518.

Paine-Murrieta GD, Taylor CW, Curtis RA, Lopez MH, Dorr RT, Johnson CS, Funk CY, Thompson F, Hersh EM. 1997. Human tumor models in the severe combined immune deficient (scid) mouse. Cancer Chemoter. Pharmacol. 40:209-214.

Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78:773-85.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. 1997a. The receptor for the cytotoxic ligand TRAIL. Science 276:111-113.

Pan G, Ni J, Wei YF, Yu G, Genty R, Dixit VM. 1997b. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 277:815-818.

Paolieri F, Salmaso C, Battifora M, Montagna P, Pesce G, Bagnasco M, Richiusa P, Galluzzo A, Giordano C. 1999. Possible pathogenetic relevance of interleukin-1 beta in "destructive" organ-specific autoimmune disease (Hashimoto's thyroiditis). Annals of the New York Academy of Sciences 876:221-228.

Remy JJ, Salamero J, Michel-Bechet M, Charreire J. 1987. Experimental autoimmune thyroiditis induced by recombinant interferon-γ. Immunol. Today 8:73.

Resetkova E, Nishikawa M, Mukuta T, Arreaza G, Fornasier VL, Volpé R. 1995. Homing of ^{51}Cr-labeled human peripheral lymphocytes to Graves' thyroid tissue xenografted into SCID mice. Thyroid 5:293-298.

Roura-Mir C, Catalfamo M, Sospedra M, Alcalde L, Pujol-Borrell R, Jaraquemada D. 1997. Single-cell analysis of intrathyroidal lymphocytes shows differential cytokine expression in Hashimoto's and Graves' disease. Eur. J. Immunol. 27:3290-3302.

Rudin CM, Thompson CB. 1997. Apoptosis and disease: regulation and clinical relevance of programmed cell death. Ann.Rev. Med. 48: 267-281.

Sack J, Baker JR, Weetman AP, Wartofsky L, Burman KD. 1987. Thyrocyte specific killer cell activity is decreased in patients with thyroid carcinoma. Cancer 59:1914-1917.

Scaffidi C, Kirchhoff S, Krammer PH, Peter ME. 1999. Apoptosis signaling in lymphocytes. Curr. Opin. Immunol. 11: 277-285.

Schultes BC, Zhang C, Xue LY, Noujaim AA, Madiyalakan R. 1999. Immunotherapy of human ovarian carcinoma with OvaRex Mab-B43.13 in human–PBL-SCID/BG mouse model. Hybridoma 18:47-55.

Schuppert F, Hiller W, Weinland G, Hoang-Vu C, von zur Muhlen A, Kohn LD. 1996. Iodide, cytokines and TSH-receptor expression in Graves' disease. Exp. Clin. Endocrinol. Diab. 104:68-74.

Sedger LM, Shows DM, Blanton RA, Peschon JJ, Goodwin RG, Cosman D, Wiley SR. 1999. IFN-γ mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. J. Immunol. 163:920-926.

Siegel RM, Fleisher TA. 1999. The role of Fas and related death receptors in autoimmune and other disease states. J. Allerg. Clin. Immunol. 103:729-738.

Snapper CM, Mond JJ. 1993. Towards a comprehensive view of immunoglobulin class switching. Immunol. Today 14:15-17.

Song K, Chen Y, Goke R, Wilmen A, Seidel C, Goke A, Hilliard B, Chen Y. 2000. Tumour Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Is an Inhibitor of Autoimmune Inflammation and Cell Cycle Progression. J. Exp. Med. 191:1095-1103.

Sreelekha TT, Pradeep VM, Vijayalakshmi K, Belthazar A, Chellam VG, Nair B, Pillai MR. 2000. In situ apoptosis in the thyroid. Thyroid 10:117-122.

Stalder T, Hahn S, Erb P. 1994. Fas antigen is the major target molecule for $CD4^+$ T cell-mediated cytotoxicity. J. Immunol. 152:1127-1133.

Stassi G, Di Liberto D, Todaro M, Zeuner A, Ricci-Vitiani L, Stoppacciaro A, Ruco L, Farina F, Zummo G, De Maria R. 2000. Control of target cell survival in thyroid autoimmunity by T helper cytokines via regulation of apoptotic proteins. Nat. Immun. 1:483-488.

Studer H, Derwahl M. 1995. Mechanisms of nonneoplastic endocrine hyperplasia - a changing concept: a review focused on the thyroid gland. Endocr. Rev. 16:411-426.

Stull SJ, Sharp GC, Kyriakos M, Bickel JT, Braley-Mullen H. 1992. Induction of granulomatous experimental autoimmune thyroiditis in mice with *in vitro* activated effector T cells and anti-IFN-γ antibody. J. Immunol. 149:2219-2226.

Su X, Hu Q, Kristan JM, Costa C, Shen Y, Gero D, Matis LA, Wang Y. 2000. Significant role for Fas in the pathogenesis of autoimmune diabetes. J. Immunol. 164:2523-2532.

Suda T, Okayaki T, Naito Y, Yokota T, Arai N, Ozaki S, Nakao K, Nagata S. 1995. Expression of Fas ligand in cells of T cell lineage. J. Immunol. 154:3806-3813.

Suda T, Nagata S. 1997. Why do defects in the Fas-Fas ligand system cause autoimmunity? J. Allerg. Clin. Immunol. 100:S97-101.

Tam YK, Miyagawa B, Ho VC, Klingemann HG. 1999. Immunotherapy of malignant melanoma in a SCID mouse model using highly cytotoxic natural killer cell line NK-92. J. Hematother. 8:281-290.

Tamura M, Kimura H, Koji T, Tominaga T, Ashizawa K, Kiriyama T, Yokoyama N, Yoshimura T, Eguchi K, Nakane PK, Nagataki S. 1998. Role of apoptosis of thyrocytes in a rat model of goiter. A possible involvement of Fas system. Endocrinology 139:3646-3653.

Tang H, Mignon GK, Meroni PL, Garotta G, Charreir J, Nicoletti F. 1993. The effects of a monoclonal antibody to interferon-γ on experimental autoimmune thyroiditis (EAT): prevention of disease and decrease of EAT-specific T cells. Eur. J. Immunol. 23:275-278.

Tang H, Chen K, Wei Y, Sharp GC, McKee L, Braley-Mullen H. 2000. Apoptosis of thyrocytes and effector cells during induction and resolution of granulomatous experimental autoimmune thyroiditis. Int. Immunol. 12:1629-1639.

Tanimoto C, Hirakawa S, Kawasaki H, Hayakawa N, Ota Z. 1995. Apoptosis in thyroid diseases: a histochemical study. Endocrine J. 42:193-201.

Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456-1462.

Van Valen F, Fulda S, Truckenbrod B, Eckervogt V, Sonnemann J, Hillmann A, Rodl R, Hoffmann C, Winkelmann W, Schafer L, Dockhorn-Dworniczak B, Wessel T, Boos J, Debatin KM, Jurgens H. 2000. Apoptotic responsiveness of the Ewing's sarcoma family of tumours to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Int. J. Cancer 88:252-259.

Vaux DL, Korsmeyer SJ. 1999. Cell death in development. Cell 96:245-254.

Vladutiu AO, Rose NR. 1971. Autoimmune murine thyroiditis relation to histocompatibility (H-2) type. Science 174:1137-1139.

Volpé R, Kasuga Y, Akasu F, Morita T, Yoshikawa N, Resetkova E, Arreaza G. 1993. The use of the severe combined immunodeficient mouse and the athymic “nude” mouse as models for the study of human autoimmune thyroid disease. Clin. Immunol. Immunopathol. 67:93-99.

Volpé R. 1996. Graves’ disease /model of SCID mouse. Exp. Clin. Endocrinol. Diabetes 104:37-40.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nature Med. 5:157-63.

Walczak H, Krammer PH. 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp. Cell. Res. 256:58-66.

Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, Yao X, Puck JM, Straus SE, Lenardo MJ. 1999. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell 98:47-58.

Weetman AP, Rees AJ. 1988. Synergistic effects of recombinant tumor necrosis factor and interferon-γ on rat thyroid cell growth and Ia antigen expression. Immunology 63:285-289.

Weigle WO. 1980. Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. Adv. Immunol. 30:159-273.

Wendling U, Walczak H, Dorr J, Jaboci C, Weller M, Krammer PH, Zipp F. 2000. Expression of TRAIL receptors in human autoreactive and foreign antigen-specific T cells. Cell. Death Differ. 7:637-644.

Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA, Goodwin RG. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3:673-682.

Williams SS, Alosco TR, Croy BA, Bankert RB. 1993. The study of human neoplastic disease in severe combined immunodeficient mice. Lab. Anim. Sci. 43:139-146.

Yamada K, Takane-Gyotoku N, Yuan X, Ichikawa F, Inada C, Nonaka K. 1996. Mouse islet cell lysis mediated by interleukin-1-induced Fas. Diabetologia 39:1306-1312.

Yasukawa M, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. 2000. Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific $CD4^+$ as well as $CD8^+$ cytotoxic T lymphocytes in humans. Blood 95:2352-2355.

Zhang XD, Nguyen T, Thomas WD, Sanders JE, Hersey P. 2000a. Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. FEBS Lett. 482:193-199.

Zhang XD, Franco AV, Nguyen T, Gray CP, Hersey P. 2000b. Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. J. Immunol. 164:3961-3970.

List of publications related to the Ph.D. thesis

1. Bretz JD, Mezosi E*, Giordano TJ, Gauger PG, Thompson NW, Baker JR, Jr. 2002. Inflammatory cytokine regulation of TRAIL-mediated apoptosis in thyroid epithelial cells. Cell Death Differ. 9:274-286.

*The first two authors contributed equally to this manuscript

2. Wang SH, Bretz JD, Phelps E, Mezosi E, Arscott PL, Utsugi S, Baker JR, Jr. 2002. A unique combination of inflammatory cytokines enhances apoptosis of thyroid follicular cells and transforms nondestructive to destructive thyroiditis in experimental autoimmune thyroiditis. J. Immunol. 168:2470-2474.

3. Mezosi E, Yamazaki H, Bretz JD, Wang SH, Arscott PL, Utsugi S, Gauger PG, Thompson NW, Baker JR, Jr. 2002. Aberrant apoptosis in thyroid epithelial cells from goiter nodules. J. Clin. Endocrinol. Metab. (accepted for publication)

4. Mezosi E, Gyory F, Szakall S, Bajnok L, Juhasz I, Szabo J, Leovey A, Kakuk G, Lukacs G, Nagy EV. Establishment of the hu-PBL-SCID mouse model for the investigation of thyroid cancer. Cancer Immunol. Immunother. (submitted)

List of other publications

1. Szabó J, Fórizs E, Bakó G, Mezősi E, Leövey A. 1987. Detectability of antibodies directed against different thyroid sructures by indirect immunfluorescence with Graves' patients' sera. A prospective study. Radiobiol. Radiother. 28:600-607.

2. Szabó J, Márián T, Balkay L, Mezősi E, Trón L. 1994. Short term effect of TSH on the intracellular pH of porcine thyroid cells in suspension. Exp. Clin. Endocrinol. (Life Sci. Adv.) 13:19-23.

3. Szabó J, Fóris G, Paragh G, Sztojka I, Mezősi E. Leövey A. 1996. Parameters of respiratory burst and arachidonic acid metabolism in polymorphonuclear granulocytes from patients with various thyroid diseases. Exp. Clin. Endocrinol. Diabetes 104:172-176.

4. Nagy E, Mezősi E, Jenei Z, Leövey A. 1996. A pajzsmirigy betegségeinek molekuláris biológiája. Orv. Hetil. 137: 563-568

5. Berczi Cs, Mezősi E, Lukács G, Balázs Gy. 1997. A preoperativ lokalizációs eljárások jelentősége a primer hyperparathyreosis miatt végzett parathyreoidectomiákban. Magyar Sebészet 50:195-198.

6. Boda J, Paragh G, Szabó J, Mezősi E, Nagy E. 1997. Lipoprotein (a) vizsgálatok pajzsmirigy betegségekben. Orv. Hetil. 138(36 Suppl 2):2307-2309.

7. Szabo J, Foris G, Keresztes T, Csabina S, Varga Zs, Bako Gy, Mezosi E, Nagy E, Paragh G, Leovey A. 1998. Heterogenous signal pathways through TSH receptors in porcine thyroid cells following stimulation with Graves' immunoglobulin G. Eur. J. Endocrinol. 139:355-358.

8. Bajnok L, Varga J, Mezősi E, Nagy E, Szabó J, Sztojka I, Galuska L, Leövey A. 1998. A hyperthyreosis radiojód terápiájának szempontjai. Orvosképzés 77:91-98.

9. Mezosi E, Bajnok L, Gyory F, Varga J, Sztojka I, Szabo J, Galuska L, Leovey A, Kakuk Gy, Nagy E. 1999. The role of technetium-99m methoxyisobutylisonitrile scintigraphy in the differential diagnosis of cold thyroid nodules. Eur. J. Nucl. Med. 26:798-803.

10. Bajnok L, Mezosi E, Nagy E, Szabo J, Sztojka I, Varga J, Galuska L, Leovey A. 1999. Calculation of the raioiodine dose for the treatment of Graves' hyperthyroidism: Is more than seven-thousand rad target dose necessary? Thyroid 9:865-869.

11. Nagy EV, Mezősi E, Burch HB, Koncz Á, Zilahi Zs, Bundik Zs. and Leövey A. 1999. Az endocrin ophthalmopathia pathogenesise: új orbita-autoantigén azonosítása nyúlban termelt TSH-receptor elleni antitestekkel. Magy. Belorv. Arch. 52:281-284.

12. Gyory F, Lukacs G, Juhasz F, Mezosi E, Szakall S, Vegh T, Math J, Balazs G. 1999. Surgically treated Hashimoto's thyroiditis. Acta Chir. Hung. 38(3-4):243-247.

13. Mezosi E, Molnar I, Jakab A, Balogh E, Karanyi Z, Pakozdy Z, Nagy P, Gyory F, Szabo J, Bajnok L, Leovey A, Kakuk G, Nagy EV. 2000. Prevalence of iodine deficiency and goitre during pregnancy in east Hungary. Eur. J. Endocrinol. 143(4):479-83.

14. Nagy EV, Toth J, Kaldi I, Damjanovich J, Mezosi E, Lenkey A, Toth L, Szabo J, Karanyi Z, Leovey A. 2000. Graves' ophthalmopathy: eye muscle involvement in patients with diplopia. Eur. J. Endocrinol. 142(6):591-597.

15. Győry F, Lukács G, Nagy EV, Juhász F, Mezősi E, Szakáll S, Máth J, Balázs G. 2001. Differenciált pajzsmirigyrák: prognosztikai faktorok vizsgálata. Magyar Sebészet 54(2):69-74.

16. Berczi C, Mezosi E, Galuska L, Varga J, Bajnok L, Lukacs G, Balazs G. 2002. Technetium-99m-sestamibi/pertechnetate subtraction scintigraphy vs ultrasonography for preoperative localization in primary hyperparathyroidism. Eur. Radiol. 12(3):605-609.

Chapters in books

1. Mezősi E, Szücs S, Sárközi S, Fórizs E, Szabó J, Leövey A. 1992. The effect of anti-microsomal antibodies on thyroid peroxidase activity. In: "New Aspects in Thyroid Disease" Medullary Thyroid Carcinoma - Thyroiditis - Peripheral Thyroid Hormone Metabolism. Deckart HF, Strehlau E. Eds. Walter de Gruyter, Berlin-New York, pp. 15-24.

2. Fórizs E, Penyige A, Jenei A, Fülöp T, Szabó J, Mezősi E, Seres I, Leövey A. 1992. Guanyl nucleotide regulatory proteins and GTP-ase activity in normal and hyperthyroid human thyroid tissue and polymorphonuclear granulocytes. In: "New Aspects in Thyroid Disease" Medullary Thyroid Carcinoma - Thyroiditis - Peripheral Thyroid Hormone Metabolism. Deckart HF, Strehlau E. Eds. Walter de Gruyter, Berlin-New York, pp. 53-60.

3. Bakó G, Nagy E, Fórizs E, Szabó J, Karányi Zs, Mezősi E, Leövey A. 1994. Changes of level of thyroid autoantibodies (TBII, Tsab, anti-HTG, anti-TPO) during metimazole treatment of patients with Basedow′s disease In: Schilddrüse 1993. Therapie der Hyperthyreose. Eds: Reinwein D. Weinheimer B, W. de Gruyter, Berlin-New-York. pp. 85-90.

4. Mezősi E. 2001. Terhesség és endocrin kórképek. In: A klinikai endokrinológia és anyagcsere-betegségek kézikönyve, Ed.: Prof. Leövey András. Medicina Könyvkiadó Rt. Budapest, 530-535.

5. Mezősi E. Daganatos betegségek és a vese. In: Klinikai nephrológia, Ed.: Prof. Kakuk György. (in press).

Abstracts

1. Fórizs E, Szücs S, Szabó J, Mezősi E, Leövey A. Detectability of autoantibodies to thyroid peroxidase in patients with autoimmune thyroid disease using highly purified porcine thyroid peroxidase. (Abstr.). Exp. Clin. Endocrinol. 97:365, 1991.

2. Szabó J, Fórizs E, Szücs S, Mezősi E, Hampel R, Leövey A. The effect of TSH, TSI, TGI on cultured porcine thyroid cells. The suitability of this system. (Abstr.) Exp. Clin. Endocrinol. 97:395, 1991.

3. Bajnok L, Mezosi E, Nagy E, Szabo J, Sztojka I, Varga J, Galuska L, Leovey A. Is the optimum dose for the elimination of Graves' hyperthyroidism linear to the gland mass? (Abstr.) Thyroid 7(Suppl.1.):S-28, 1997.

4. Mezosi E, Bajnok L, Gyory F, Varga J, Szabo J, Leovey A, Nagy E. The role of technetium-99m-methoxyisobutylisonitrile (MIBI) scintigraphy in the differential diagnosis of cold thyroid nodules. (Abstr.) Thyroid 7(Suppl.1.):S-81, 1997.

8. ACKNOWLEDGMENT

I want to express many thanks to Endre V. Nagy, my supervisor, for supporting me at every stage of this work, James Baker Jr. for the opportunity to do research in his lab and for calling my attention on the importance of apoptosis in the pathogenesis of thyroid diseases. I am grateful to György Kakuk allowing me to participate in the Ph.D. program and visiting research fellow program, György Paragh for his recommendations and useful discussions and András Leövey who motivated me to be an internist and endocrinologist. Thanks a lot to Zsuzsa Varga for the correction of the Ph.D. thesis and for the help she provided as a researcher and friend. Many thanks to my coworkers, first of all Ferenc Gyory, from whom I learned persistence, friendship and humanity, Jenő Szabo, my first tutor, who supported my work from the beginning, Su He Wang, Patty Arscott, Hiroko Yamazaki, Saho Utsugi and James Bretz who taught me for the laboratory technics and apoptosis studies, encouraged and helped me in everything during the research fellowship, Zsolt Karányi for directing me in the statistical analysis, Szabolcs Szakáll whom I could ask any time for advice, István Juhász who made the studies on SCID mice possible. I am grateful to Sallai Csabáné, Lénárt Ferencné and Herbály Mihályné for technical assistance. Many thanks to my family who accompanied me to the US, especially to my husband, László Bajnok who was my first advisor and helped to revise all my papers.