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A Unique Combination of Inflammatory Cytokines Enhances Apoptosis of Thyroid Follicular Cells and Transforms Nondestructive to Destructive Thyroiditis in Experimental Autoimmune Thyroiditis[1]

Su He Wang, James D. Bretz, Ellen Phelps, Emese Mezosi, Patricia L. Arscott, Saho Utsugi, and James R. Baker, Jr.[2]

Treatment of cultured primary human thyroid cells with IFN-γ and TNF-α uniquely allows the induction of Fas-mediated apoptosis. To investigate the role of this cytokine combination in vivo, CBA/J mice were immunized with thyroglobulin and then injected with IFN-γ and TNF-α. Compared with control animals, mice treated with IFN-γ and TNF-α showed significantly sustained lymphocytic infiltration in the thyroid, which was associated with the destruction of portions of the follicular architecture at wk 6 after initial immunization. Furthermore, the number of apoptotic thyroid follicular cells was increased only in the thyroids from mice treated with the IFN-γ and TNF-α. We also analyzed the function of the Fas pathway in vivo in cytokine-treated mice by using an agonist anti-Fas Ab injected directly into the thyroid. Minimal apoptosis of thyroid epithelial cells was observed unless the mice were pretreated with IFN-γ and TNF-α. These data demonstrate that this unique combination of inflammatory cytokines facilitates the apoptotic destruction of thyroid follicular cells in experimental autoimmune thyroiditis, in a manner similar to what is observed in Hashimoto's thyroiditis in humans. *The Journal of Immunology,* 2002, 168: 2470–2474.

T hyroiditis can be experimentally induced in mice bearing the H-2^K haplotype by immunization with thyroglobulin (Tg)[3] and adjuvants (1). In this experimental autoimmune thyroiditis (EAT) model, mice develop autoimmune responses characterized by the occurrence of circulating anti-Tg Abs 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 (HT), 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 (2, 3).

Apoptosis is a mechanism of physiological cell elimination that induces cells to self-destruct when stimulated by an appropriate trigger, and improper regulation of apoptosis can lead to disease (4, 5). It can be initiated in cells through endogenous molecules such as TNF and Fas ligand (FasL). Apoptosis appears to play a role in the pathogenesis of autoimmune thyroiditis in humans (6, 7). Normal thyroids show a low level of apoptosis, a possible result of basal thyroid cell turnover (6, 7). In contrast, thyroid cells from patients with autoimmune thyroiditis display an increased frequency of apoptotic cells (6–9). Many of the apoptotic cells in

these glands are found in the areas of disrupted follicles, in proximity to infiltrating lymphoid cells (9, 10). 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.

It has been shown that inflammatory cytokines are involved in the regulation of apoptosis (11). Cytokines can regulate the expression of apoptotic signaling components and inhibitors in target cells, as well as control the expression of apoptotic initiators in effector cells (12). 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 (13–16). It has been shown that T cell clones isolated from intrathyroidal lymphocytic infiltrates of HT produce high levels of IFN-γ and TNF-α (17, 18), and cytokine-producing lymphocytes also are observed adjacent to thyrocytes (19). Furthermore, IFN-γ in combination with TNF-α impairs the growth of thyrocytes (20), and previous studies from our laboratory have shown that the pretreatment of primary normal human thyroid cells with the combination of IFN-γ and TNF-α allowed the induction of Fas-mediated apoptosis in vitro (12). Several publications have suggested a crucial role for cytokines in pathogenesis of EAT (13–16), but the exact function of these molecules is not clear. There has also been no information on the effect of the administration of both IFN-γ and TNF-α on the thyroid in vivo. We used the EAT model to test the influence of the in vivo administration of IFN-γ and TNF-α and show data suggesting that these cytokines can facilitate thyroid destruction in thyroiditis.

Materials and Methods
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 wk before

initiating the experiment. Mice were s.c. challenged at the base of the tail with 100 μg of porcine Tg (pTg; Sigma-Aldrich, St. Louis, MO) emulsified in CFA (Difco, Detroit, MI). Two weeks later, the mice were boosted with the same dose of pTg in incomplete Freund's adjuvant. Control animals were injected with 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 rIFN-γ (R&D Systems, Minneapolis, MN), 0.5 μg of mouse rTNF-α (R&D Systems), or 5 μg of IFN-γ and 0.5 μg of TNF-α in PBS for 3 consecutive days. Control animals were injected with BSA in PBS.

Serum anti-Tg Ab quantitation

Anti-Tg Ab was assayed by solid-phase ELISA. Serial dilutions of sera collected before immunization, 2 wk after initial immunization, and before sacrifice were used to determine endpoint titers. Briefly, round-bottom 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, West Grove, PA) was added as second Ab, 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, Rockford, IL), then run on SDS-polyacrylamide gel, and immunoblot analysis was performed with mouse serum (diluted 1/4,000) prepared from pTg-immunized mice treated with or without cytokines. A commercial rabbit anti-Tg (DAKO, Carpenteria, CA) was used as a positive control.

Thyroid histopathology

Thyroid glands were fixed in 10% formalin, embedded in paraffin, or directly frozen in Tissue-Tek OCT (Sakura Finetechnical, Nihombashi, Chuo-ku, Tokyo) and sectioned by standard method. Infiltration was evaluated on 5-μm-thick sections stained with H&E. The severity of thyroiditis was graded on a scale of 0–4, as follows (21): 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.

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, Purchase, NY), 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, San Francisco, CA) and incubated with biotin-conjugated rat monoclonal anti-mouse CD45 (Ly-5), CD4 (L3T4), or CD8 (Ly-2) Abs or isotype-matched rat IgG2b or IgG2a (BD PharMingen, San Diego, CA). Then, the sections were incubated with streptavidin-peroxidase conjugate and were developed by incubation with diaminobenzidine substrate. The slides were counterstained with hematoxylin or methyl green and mounted with GVA MOUNT (Zymed Laboratories).

Thyroid injection with anti-Fas Ab

For direct injection of Ab 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 IFN-γ and 0.5 μg of TNF-α or BSA in PBS for 3 consecutive days before Ab 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 (Reno, NV). 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.

Statistical analysis

All values were expressed as mean ± SE. The relationships among variables were analyzed with the Wilcoxon matched pairs test using Stat View software (Abacus Concepts, Berkeley, CA). A *p* value of <0.05 was taken as statistically significant.

Results

The combination of IFN-γ and TNF-α enhances lymphocytic infiltration induced by pTg

CBA/J mice were immunized with heterologous Tg in CFA to evaluate the effect of IFN-γ and TNF-α in murine EAT. IFN-γ and TNF-α, either singly or together, were injected into mice on wk 3 after Tg immunization. On wk 4 and 6 after initial immunization, thyroid glands were collected for histopathological studies. As expected for this model, animals immunized with Tg showed mononuclear cell infiltration by 4 wk 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. 1*a*). In contrast, 6 wk post-pTg immunization, thyroids from mice treated with pTg and IFN-γ/TNF-α showed markedly sustained mononuclear cell infiltration compared with thyroids from mice without cytokine treatment ($p < 0.01$; Fig. 1*b*). Thyroids from mice treated with pTg and TNF-α showed increased mononuclear cell infiltration compared with thyroids from pTg alone mice, but less than that observed with the combination of two cytokines. The destruction of portions of the follicular architecture was observed in IFN-γ/TNF-α-treated mice (Fig. 2), but not in the animals immunized with either Tg alone or each cytokine singly.

Characterization of infiltrating cells

To identify the phenotype of cellular inflammation in the thyroid, we stained mice thyroid sections with CD45 Ab, which identifies leukocytes. Fig. 3 (*upper right*) identified the infiltrating mononuclear cells in the thyroid as primarily leukocytes. Further staining with CD4 and CD8 Abs showed that both CD4$^+$ and CD8$^+$ T cells were present in thyroids from mice treated with pTg and IFN-γ
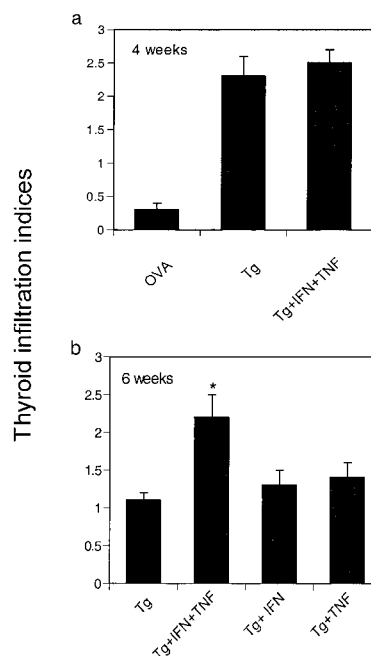

FIGURE 1. Lymphocytic infiltration indices of murine thyroid glands. CBA/J mice were injected with the immunogen (OVA or pTg) and cytokines or BSA control, as described in *Materials and Methods*. Mice were sacrificed, and the lymphocytic infiltration was quantified at 4 (*a*) or 6 (*b*) wk postimmunization. Values represent the mean ± SEM of 9–12 animals per group. *, $p < 0.01$ compared with Tg immunization alone.
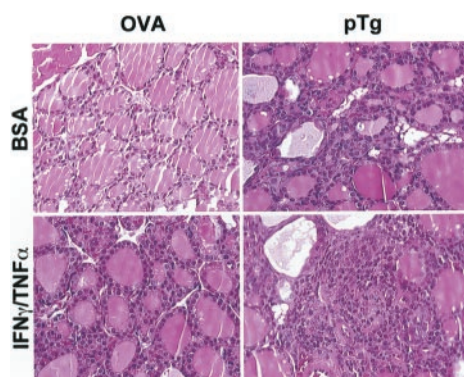
FIGURE 2. Histology of thyroid glands. Thyroid sections were prepared from mice at wk 6 after initial immunization. Treatments of mice before sacrifice are indicated as follows: immunized with OVA (*upper left*); immunized with OVA, then administrated IFN-γ + TNF-α (*lower left*); immunized with pTg (*upper right*); immunized with Tg, then given IFN-γ + TNF-α (*lower right*).

FIGURE 4. In situ TUNEL staining of EAT thyroids to identify apoptotic cells. Sections from mice 6 wk after initial immunization with pTg and treated with control protein (*upper panel*) vs those immunized with pTg and treated with IFN-γ + TNF-α (*lower panel*).

and TNF-α, and these findings were similar to those observed in animals immunized with Tg alone (data not shown).

Analysis of apoptosis in EAT mice treated with IFN-γ and TNF-α

Tg-induced autoimmunity in this model has not previously resulted in a destructive thyroiditis, although recent reports (22, 23) have demonstrated that cytokine treatments can alter the pathology of EAT from a lymphocytic to a granulomatous form. To determine whether IFN-γ and TNF-α treatment specifically promotes thyrocyte destruction, thyroids from EAT mice were evaluated for cell death by in situ TUNEL staining. On wk 6 after initial immunization, the number of apoptotic cells was markedly increased in the thyroids of EAT mice treated with the combination of IFN-γ and TNF-α, compared with mice without cytokine treatment. This was demonstrated by the number of TUNEL-positive cells shown in the *lower panel* of Fig. 4. Our prior work suggested that alterations of the Fas pathway were involved in the ability of TNF-α and IFN-γ to facilitate apoptosis in thyroid cells (12). 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 Ab directly into the thyroid. The number of apoptotic thyroid epithelial cells was markedly increased in the thyroids of mice pre-
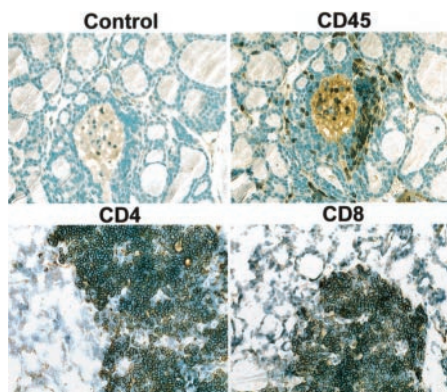
treated with IFN-γ/TNF-α compared with mice treated with Fas agonist alone (Fig. 5).

Humoral response to Tg

To ascertain whether the cytokine treatment and associated lymphocyte infiltration and thyrocyte apoptosis altered the humoral immune response, we determined the titer of Abs to the immunogen pTg. Titers of anti-Tg Abs were determined in sera from pTg- and OVA-immunized mice with or without cytokine treatment, and this was compared with preimmune serum. As expected, the titers of anti-Tg Ab were significantly increased in all pTg-immunized mice, while control mice injected with OVA had no evidence of anti-Tg Ab regardless of cytokine treatment. However, the titers of anti-pTg Ab from sera of pTg-immunized mice treated with or without cytokines did not differ significantly (data not shown). IFN-γ has a demonstrated role in modulating isotype switching (24), and to examine this effect in the EAT model, subclasses of Ab to pTg were determined in sera from immunized mice treated with or without cytokine. The titers of IgG1 and IgG2a anti-Tg Abs were similar in sera from Tg-immunized mice regardless of cytokine treatment (data not shown). We also examined the mouse

FIGURE 3. CD4, CD8, CD45, and control Ab immunohistochemical staining of thyroid sections. Thyroid sections are from mice immunized with pTg and treated with IFN-γ + TNF-α.

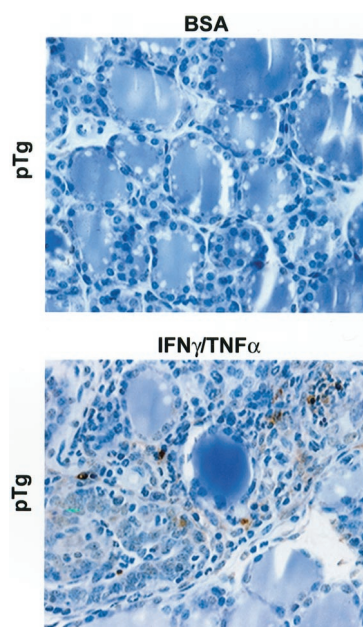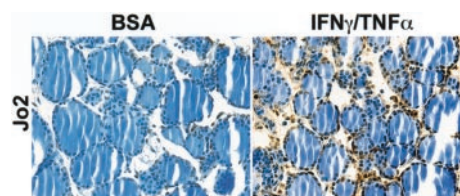**FIGURE 5.** Functional analysis of the Fas pathway in vivo. Mice without immunization were treated with the agonist anti-Fas Ab (Jo2) injected directly into the thyroid, and apoptosis was determined by TUNEL staining. Thyroid sections that we prepared from mice injected with Jo2 alone (*left panel*) or with Jo2 after pretreatment with both IFN-γ and TNF-α (*right panel*).

serum Abs 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 Ab (data not shown).

Discussion

The hallmark of chronic thyroiditis in humans is the disruption of thyroid follicular structure with progressive glandular disruption leading to hypofunction (1, 2). Immune mediated events underlie this process, and recent studies suggest that apoptosis mediates at least a portion of the follicular cell cytotoxicity (6, 7). 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 Ag 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 (13). In contrast to spontaneous thyroiditis in humans and animals, EAT is self-limited, resolving over several weeks, and does not result in follicular disruption (13). 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, IFN-γ and TNF-α, 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 Abs 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 immunized Tg. These data also suggest that the Ab response alone is not adequate to mediate apoptotic disruption of the thyroid.

The role that IFN-γ and TNF-α 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 from our laboratory demonstrate that the unique combination of IFN-γ and TNF-α facilitates Fas-mediated apoptosis in primary human thyrocytes in vitro (12), while a double dose of IFN-γ or TNF-α 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 (25). In the current studies, injection of anti-Fas Ab into the thyroids of mice yielded apoptosis only in animals pretreated with IFN-γ and TNF-α. 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 IFN-γ administered alone on the pathogenesis of autoimmune thyroiditis are still not clear. Experiments by Remy et al. (26) induced EAT-like infiltrates, but not cytolysis through intrathyroidal injection of IFN-γ, and the application of IFN-γ-neutralizing Ab appears to retard the development of EAT (27). However, others have reported that the neutralization of endogenous IFN-γ exacerbates granulomatous EAT (28). IFN-γ is also not required for the experimental induction of autoimmune thyroiditis, as traditional pathologic changes of EAT can occur in mice with disrupted *IFN-γ* or *IFN-rR* genes (13). In contrast, synergy between IFN-γ and TNF-α has only been reported in the regulation of the Fas pathway (29, 30) and can reduce Fas pathway inhibitors that are normally present in the thyroid (31). Thus, the induction of destructive thyroiditis in EAT is not simply the result of independent inflammatory actions of either IFN-γ or TNF-α, 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 (32), 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 humans.

The mechanism of cytotoxicity in thyroiditis may also be important in generating cytotoxicity. CTL kill their target cells predominantly through granule exocytosis with perforin/granzyme and/or the Fas-FasL system (33, 34), with the Fas-FasL pathway operative in both CD4$^+$ and CD8$^+$ T cells (25, 35–37). Although granule exocytosis-mediated CTL against thyroid follicular cells might play a role in the destruction of HT thyrocytes (38), increasing evidence suggests that the Fas-FasL pathway is central to this process (31). Because FasL is present on activated T lymphocytes (39, 40) and the lymphocytic infiltration of the thyroid of IFN-γ- and TNF-α-treated EAT includes both CD4$^+$ and CD8$^+$ T effector cells (25), Fas on the surface of thyrocytes should mediate thyroid apoptosis. However, prior studies we have conducted indicate that this pathway is normally blocked in thyroid cell (12, 31), and 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 IFN-γ and TNF-α, but it is unclear what mechanism is used by the two cytokines to influence the Fas pathway. Our previous study demonstrated that susceptibility to Fas-induced death by the combination of IFN-γ and TNF-α in thyroid cell correlated with an increase in expression of a tunicamycin-inhibitable high m.w. form of Fas (12). This strongly suggested that the 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 for the induction of apoptosis and thyroid disruption in EAT.

In summary, the combination of IFN-γ and TNF-α 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 IFN-γ and TNF-α and increased thyrocyte apoptosis (25). 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 Fas-associated death domain protein-like interleukin 1β-coverting enzyme-inhibitory protein or other Fas regulatory proteins may predispose individuals to these disorders.

Acknowledgments

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