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The Thyrotropin (Thyroid-Stimulating Hormone) Receptor Is Expressed on Murine Dendritic Cells and on a Subset of CD45RB^{high} Lymph Node T Cells: Functional Role for Thyroid-Stimulating Hormone During Immune Activation¹

E. Ümit Bağrıaçık and John R. Klein²

Thyroid-stimulating hormone (TSH), a central neuroendocrine mediator of the hypothalamus-pituitary-thyroid axis, has been shown to affect various aspects of immunological development and function. To gain a better understanding of TSH involvement within the mammalian immune system, the expression and distribution of the TSH receptor (TSHr) has been studied by immunoprecipitation and by flow cytometric analyses. Using highly enriched populations of B cells, T cells, and dendritic cells, trace amounts of TSHr were precipitated from B cells and T cells, whereas high levels of TSHr were precipitated from the dendritic cell fraction. Flow cytometric analyses of TSHr expression on splenic and lymph node T cells revealed a major difference between those tissues in that only 2–3% of splenic T cells were TSHr⁺, whereas 10–20% of CD4⁺8⁻ and CD4⁻8⁺ lymph node T cells expressed the TSHr, which was exclusively associated with CD45RB^{high} cells and was not expressed during or after activation. The TSHr was not present on cells of the immune system during fetal or neonatal life. However, recombinant TSHβ was found to significantly enhance the phagocytic activity of dendritic cells from adult animals and to selectively augment IL-1β and IL-12 cytokine responses of dendritic cells following phagocytic activation. These findings identify a novel immune-endocrine bridge associated with professional APCs and naive T cells. *The Journal of Immunology*, 2000, 164: 6158–6165.

The notion that the immune system and the neuroendocrine system are physiologically and functionally linked has been realized for some time and has been empirically demonstrated in a number of studies. To the present, however, most of those have examined the relationship between immune function and hormones of the hypothalamus-pituitary-adrenal axis; in particular, the involvement of the glucocorticoids and other “stress” hormones (1). Those biological mediators and their derivatives generally exert suppressive effects on the host’s immune response to Ag, and thus have been used clinically to curtail the severity of autoimmune reactions and allograft rejection responses (2–4).

The hypothalamus-pituitary-thyroid (HPT)³ axis is a neuroendocrine pathway used in development and throughout metabolic regulation. This involves an orchestrated process of hormone release and feedback involving the hypothalamic-derived neuropeptide, thyrotropin-releasing hormone (TRH); the anterior pituitary-derived hormone, thyroid-stimulating hormone (TSH); and the thyroid-derived hormones (tri-iodothyroxine (T₃) and thyroxine

(T₄)). With the exception of the direct involvement of HPT hormones in thyroid autoimmunity (reviewed in ref. 5) and thyroid-immune system interactions by hormones and cytokines (6–9), knowledge about the involvement of HPT hormones on immunity remains scant. Yet, evidence exists indicating that TRH and TSH can directly or indirectly influence peripheral immunity in a number of ways. For example, TRH has been reported to have costimulatory effects on mitogen-induced lymphocyte responses of rat spleen cells (10), and TSH has been shown to augment Ag-specific spleen plaque-forming cell responses in mice (11–13). Those apparent effects may be indirect, however, as inferred from studies demonstrating TSH binding to monocytic cells (14). Other studies have demonstrated that HPT hormones, mediated primarily by TSH, can alter the immunological composition of lymphoid cells dispersed throughout the intestinal epithelium (15–17). This occurs via a local network of TSH hormone synthesis and utilization in which TSH is produced by intestinal enterocytes and is utilized by intestinal lymphoid cells (18).

Still to be learned about the systems described above is the nature of hormone communication within the immune system, i.e., the specific cells that are involved, the molecular signals used and how they affect the overall immunobiological process, and whether additional intermediate mediators are involved in the delivery of hormone signals to target cells and tissues. In the present study, we have examined the expression of the TSH receptor (TSHr) on cells of the thymus, spleen, and lymph nodes to better understand the cells of the immune system involved in TSH utilization and to provide information about the pathways of TSH-mediated hormone communication within primary and secondary lymphoid tissues. Our findings indicate that the TSHr is expressed in a remarkably selective manner throughout cells of the peripheral immune system. The implications of this in the context of neuroendocrine homeostatic regulation of immunity are discussed.

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³ Abbreviations used in this paper: HPT, hypothalamus-pituitary-thyroid; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone (thyrotropin); TSHr, TSH receptor.

Materials and Methods

Mice and reagents

C57BL/6, BALB/c, and CB6F₁ mice used in the study were purchased from The Jackson Laboratory (Bar Harbor, ME). Data reported are from C57BL/6 mice; similar findings were obtained with BALB/c and CB6F₁ mice. C.RF-Tshr^{hyt/hyt} hypothyroid mice were raised at the University of Tulsa from breeding pairs obtained from The Jackson Laboratory. Mice homozygous for the TSHr mutation were identified by PCR analyses and DNA sequencing across the mutation region. The medullary thyroid carcinoma mouse (MTC-M) cell line (1806-CRL) was obtained from the American Type Culture Collection (Manassas, VA). Recombinant human TSH was obtained from Sigma (catalogue no. T-4533; St. Louis, MO).

Abs and flow cytometry

Abs used in this study were FITC-labeled anti-CD8 α (CT-CD8s; Caltag, South San Francisco, CA), FITC- and PE-labeled anti-CD5 (53-7.3), FITC-labeled anti-CD45RB (23G2), PE-labeled anti-CD4 (RM4-5), PE- and FITC-labeled anti-MHC-II (39-10-8), PE-labeled anti-CD69 (H1.2F3), biotin-labeled anti-CD19 (ID3), PE-labeled anti-CD11c (HL3), biotin-labeled and unlabeled anti-CD11b (MI/70), biotin-labeled and unlabeled anti-MHC-II (39-10-8), biotin-labeled anti-TCR $\alpha\beta$ (H57-597), FITC- and PE-labeled isotype/species- matched Abs for control staining, anti-CD16/32 for Fc receptor blocking before staining, and PE-streptavidin (all reagents; PharMingen, San Diego, CA). Unlabeled anti-Thy-1.2 (J1j.10), anti-CD24 (J11d.2), anti-CD4 (GK1.5), anti-CD8 (3.155), and anti-Mac1 α (MI/70) (American Type Culture Collection) were used for complement-mediated lysis. Blocking with unlabeled TSH was done by culturing 2×10^5 freshly isolated splenic dendritic cells in 100 μ l of supplemented medium with 100 μ l of 10^{-4} , 10^{-5} , or 10^{-6} M TSH for 25 min at 4°C. Cells were washed and reacted with biotin-labeled recombinant TSH for 25 min at 4°C and analyzed by flow cytometry.

Recombinant human TSH was biotinylated using *N*-hydroxysuccinimidobiotin (Sigma H1759). Thirty-five micrograms of NHS-biotin was added to 0.1 mg of recombinant TSH in 300 μ l of 0.1 M carbonate buffer (pH 9.0) and incubated for 3 h at room temperature. Four microliters of 1 M NH₄Cl was added and incubated for 15 min at room temperature. The reaction was washed twice with PBS in a Micron-10 microconcentrator (Amicon, Beverly, MA) and resuspended in 300 μ l of PBS for use.

Flow cytometric analyses was done using an Epics 751 flow cytometer (Coulter, Hialeah, FL) interfaced to a Cicero data acquisition system (Cytomation, Fort Collins, CO). All experiments included cells stained with appropriate isotype/species-matched control Abs from which the positions of the cursors were determined as shown in the histograms.

Cell isolations, enrichment, and in vitro culture

Lymphoid cells from the small intestine were isolated as described previously (19). Single cell suspensions of spleen cells, thymocytes, and lymph node cells were prepared by pressing tissues through a 60-mesh stainless steel screen. Enrichment of splenic T cells and B cells was done using published techniques with minor modifications. For T cell enrichment (20), spleen cells were incubated with anti-Mac-1 α , anti-CD24, and anti-MHC-II mAbs at 4°C for 30 min, washed, and reacted for 15 min at 37°C with 10% Low-Tox guinea pig complement (Accurate Chemicals, Westbury, NY). The complement treatment was repeated and treated cells were passed through a nylon wool column for depletion of B cells and adherent cells.

For B cell purification (21), spleen cells were collected and incubated at 1×10^8 cells/10 ml in 100-mm tissue culture plates (Fisher Scientific, Dallas, TX) at 37°C in 5% CO₂ for 1 h. Nonadherent cells were recovered and erythrocytes were lysed with 0.83% ammonium chloride. Cells were washed, layered onto a discontinuous gradient of 50, 60, 70, and 80% Percoll (Pharmacia, Uppsala, Sweden), and centrifuged for 20 min at $600 \times g$. B cells were collected from the 60 to 70% Percoll interface and residual T cells were removed by treatment with anti-Thy-1.2, anti-CD4, anti-CD8, and anti-Mac1 α Abs plus complement. Cells were centrifuged through a 50–80% Percoll gradient and cells were recovered from the 60 to 70% interface to obtain the viable B cell-enriched population.

Dendritic cell enrichment was done according to the method of Ridge et al. (22) with modifications. Briefly, spleens were isolated from 6 to 10 mice and single-cell suspensions were prepared in RPMI 1640 containing 10% FBS. Cells were incubated at a concentration of 5×10^7 cells/10 ml for 1.5 h at 37°C in tissue culture-treated petri dishes (Fisher Scientific). Plates were gently washed three to five times with warm medium followed by PBS to remove nonadherent cells. Adherent cells were then incubated at 37°C overnight in 5 ml of medium containing 5 ng/ml GM-CSF, collected by rinsing with medium and PBS, and depleted of residual T cells and B

cells by complement-mediated lysis using anti-CD3 and anti-B220 mAbs. Viable cells were collected by centrifugation over 50% Percoll. Dendritic cells and lymphocytes were recovered from day 18 fetal mice and day 7 neonatal mice. Age of fetal mice was determined by checking each morning for vaginal plugs in breeding-paired mice; plugged animals were defined as fetal day 1.

For in vitro stimulation of T cells, 60-mm petri dishes were coated with anti-CD3 Ab overnight at 4°C. Media were removed from plates and 5 ml of 3×10^6 cells/ml was cultured in RPMI 1640 supplemented with FBS (10% v/v), 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-ME (all reagents; Sigma). After 48 h, cells were collected and centrifuged over Ficoll-Paque (Sigma) at $400 \times g$ for 20 min. Viable cells from the liquid interface were collected, washed, and stained for flow cytometry.

Immunoprecipitation and Western blotting

A total of 10×10^6 freshly isolated thymocytes, whole spleen cells, fractionated spleen cells, or $3-5 \times 10^6$ MTC-M cells was lysed in detergent buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM PMSF, 1 μ g/ml aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, and 0.25% deoxycholate (all reagents; Sigma). Precleared lysates were mixed overnight with 10 μ l of monoclonal anti-TSHr Ab (clone 28) (23) (ABR, Golden, CO) at 4°C, followed by 40 μ l of protein A-agarose (Sigma) for 2 h. Precipitates were collected by centrifugation, washed, boiled in 2 \times reducing SDS sample buffer, and electrophoresed through a 10% polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred electrophoretically to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad), blocked with 3% nonfat dry milk in PBS, reacted with anti-TSHr mAb (clone 49) (23) (ABR) overnight at 4°C, followed by the addition of biotinylated anti-mouse Ig (PharMingen) for 2 h, and streptavidin-HRP (Amersham, Buckinghamshire, U.K.) for 30 min. Enhanced chemiluminescence (Amersham) was used for autoradiographic identification of proteins.

cAMP and cytokine assays

A total of 5×10^5 freshly isolated splenic dendritic cells from normal mice and C.RF-Tshr^{hyt/hyt} mice were cultured in 1 ml of unsupplemented DMEM with 1) medium alone, 2) 10^{-7} – 10^{-9} M recombinant TSH, or 3) 10^{-7} M forskolin (Sigma) in the presence of 1 mM 3-isobutyl-1-methylxanthine (Sigma), a phosphodiesterase inhibitor. After 15 min, cells were collected, pelleted by microfuge centrifugation, lysed in 0.1 M HCl, and cAMP activity was assayed using a competitive ELISA assay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Determination of cytokine activity was made using commercial cytokine assay kits according to the manufacturer's protocols (IL-1 β , IL-6, and TNF- α , R&D Systems; IL-12, Genzyme, Cambridge, MA).

Activation of dendritic cells by phagocytosis

Phagocytosis by dendritic cells was done using methods similar to those reported by others (24). A total of 5×10^5 freshly isolated splenic dendritic cells was cultured for 3 or 9 h in supplemented medium (see cell isolations) with 10 μ l of a 10% suspension of FITC-labeled latex beads (Fluoresbrite plain YG 1.0 micron microspheres; Polysciences, Warrington, PA) in the presence or absence of 10^{-8} M recombinant TSH β . Cells were recovered,

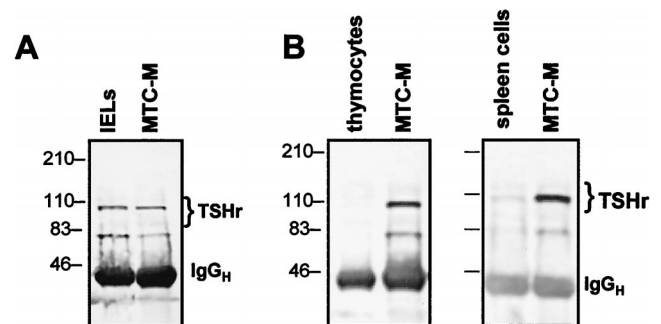


FIGURE 1. Immunoprecipitation/Western blot analyses of TSHr from intraepithelial lymphocyte lysates (A) and whole thymocytes and spleen cell lysates (B). Precipitated products obtained from the MTC-M murine thyroid cell line are shown. Numbers to the left indicate the position of protein standards markers (kDa).

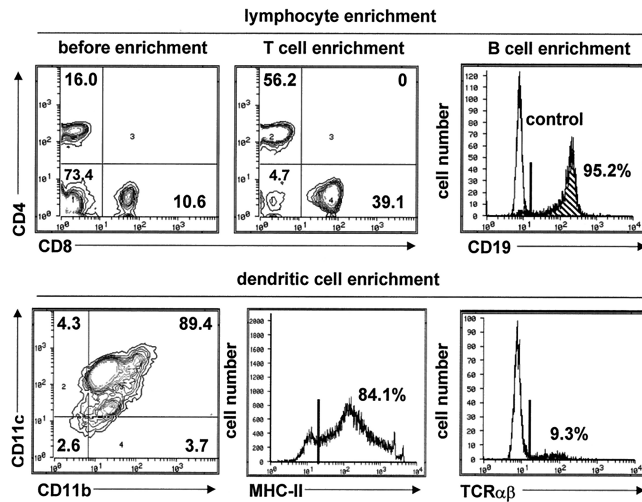


FIGURE 2. Phenotypic analyses of whole spleen cells before enrichment and after enrichment for T cells, B cells, and dendritic cells. Control staining by isotype-matched Abs are reflected by quadrant one (lower left) in two-color histograms and by the inflection mark in one-color histograms.

microfuged briefly to remove unincorporated beads, and analyzed by flow cytometry. Because of the difference in the size of cells and beads, exclusion of residual nonincorporated beads was easily done by gating onto the dendritic cell population. For zymosan activation of dendritic cells, 2×10^5 freshly isolated cells were cultured in 200 μ l of supplemented medium with 0.0025% (w/v) zymosan (ICN Pharmaceuticals, Aurora, OH) in the presence or absence of 10^{-8} M recombinant TSH β ; supernatants were collected after 18 h and assayed for cytokine activity.

Results

Immunoprecipitation of TSHr from intestinal lymphoid cells, thymocytes, and spleen cells

Cell lysates were prepared from lymphoid cells from the small intestine epithelium, from thymocytes, and from erythrocyte-depleted whole spleen cells. Immunoprecipitation and Western blot analyses were done using two anti-TSHr mAbs as described in *Materials and Methods*. Precipitation of the TSHr by this technique was confirmed using MTC-M cells which yielded a 95- to 100-kDa band characteristic of the mature glycosylated receptor protein (25) (Fig. 1). The 65- to 75-kDa band occasionally seen may reflect a subunit of the receptor protein (26, 27) or possibly nonglycosylated TSH receptor from cell lysates. As shown in Fig. 1A, TSHr-precipitated products were clearly evident in intestinal lymphoid cell preparations, confirming previous findings that the intestine is a site of high levels of TSHr expression (18). By comparison, TSHr-precipitated products were barely detectable in thy-

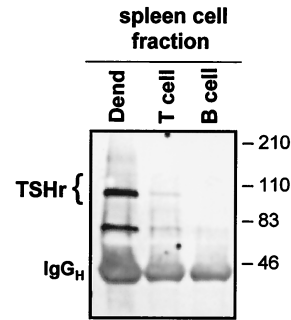


FIGURE 3. Immunoprecipitation/Western blot analyses of spleen cell fractions enriched for dendritic cells, T cells, and B cells as shown in Fig. 2. Numbers to the right indicate the position of protein standards (kDa).

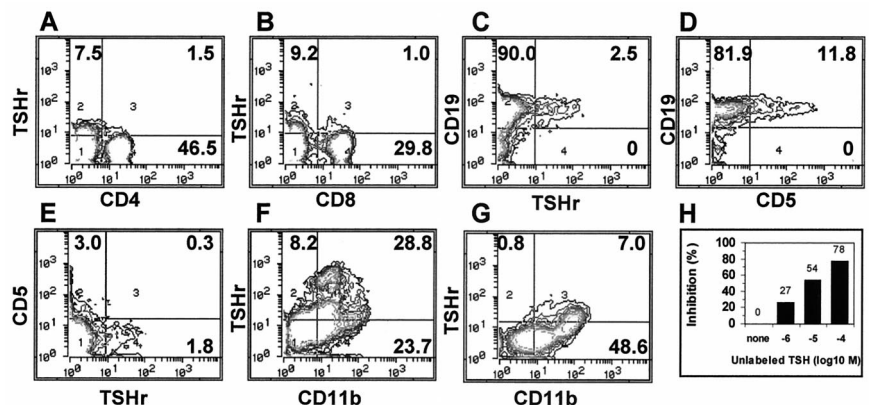
myocyte lysates and were slightly more evident in spleen cell lysates (Fig. 1B). These findings were consistent in several immunoprecipitation experiments using different mouse strains.

Within the spleen, the TSHr is preferentially expressed on dendritic cells

Experiments were done to determine whether the TSHr was expressed at low levels across all spleen cells or whether expression was linked to a particular cell subpopulation. Freshly isolated spleen cells were enriched for T cells, B cells, and dendritic cells. Fig. 2 shows the phenotypic profile of cells before and after enrichment, demonstrating 95.3% enrichment of T cells based on expression of CD4 and CD8, and 95.2% enrichment of B cells based on CD19 expression. In the dendritic cell fraction, 89.4% of the cells coexpressed CD11b and CD11c; 84.1% of the cells expressed MHC class II Ags, all of which are markers of splenic dendritic cells of the myeloid lineage (28, 29). T cell contamination of the dendritic cell-enriched fraction was <10% (Fig. 2); no contamination by B cells was observed (data not shown). Immunoprecipitation/Western blotting analyses of TSHr expression using those cells revealed minimal amounts of precipitated TSHr from B cells or T cells even after enrichment. Particularly noteworthy, however, was the finding that the TSHr was present at high levels in the dendritic cell fraction when compared with equivalent numbers of T cells and B cells (Fig. 3).

To provide more detailed information about the expression of the TSHr among lymphoid cell subsets, flow cytometric analyses were done using biotinylated recombinant TSH with splenic lymphocytes and dendritic cells. Shown in Fig. 4, A and B, only 2–3% of the CD4⁺ or CD8⁺ T cells expressed the TSHr at very low density. Similarly, only 2.7% of the total CD19⁺ B cells were TSHr⁺, although the receptor density on those cells was higher

FIGURE 4. A–F, Flow cytometric analyses of TSHr expression on splenic CD4⁺8[−] and CD4[−]8⁺ T cells, CD19-enriched B cells, CD5⁺ B cells, and CD11b⁺ splenic dendritic cells. G, Blocking of binding of biotin-labeled TSH to splenic dendritic cells using 10^{-4} M unlabeled recombinant TSH. H, Dose response of inhibition of TSH binding to splenic dendritic cells.



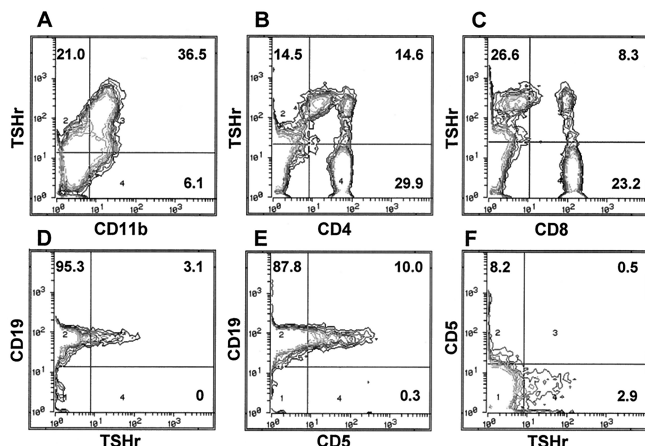


FIGURE 5. Flow cytometric analyses of TSHr expression on lymph node CD11b⁺ dendritic cells, CD4⁺8⁻ and CD4⁻8⁺ T cells, and lymph node CD19-enriched B cells and CD5⁺ B cells. Note the difference in TSHr expression on lymph node T cells compared with splenic T cells (Fig. 4).

than for T cells. Further analysis of B cells was done in conjunction with CD5, a marker of the B-1 B cell subgroup which comprises a minor but significant component of the total splenic B cells (Fig. 4D). As seen in Fig. 4E, the TSHr was expressed almost exclusively on CD5⁻ B cells, thus indicating that the small proportion of TSHr⁺ B cells is part of the general B cell pool and that, overall, the TSHr is not widely expressed on either T cells or B cells in the spleen.

Splenic dendritic cells were enriched as described above, yielding a fraction that was >80% CD11b⁺ and/or CD11c⁺ cells (data not shown). Consistent with the findings from the immunoprecipitation experiments, a significant proportion of splenic dendritic cells (37% of the cells overall and 54.8% of the CD11b⁺ cells) were TSHr⁺ (Fig. 4F). Fig. 4G demonstrates the capacity of unlabeled recombinant TSH to block the binding of biotin-labeled TSH to spleen dendritic cells; this occurred in a dose-dependent manner using unlabeled TSH (Fig. 4H). These studies, therefore, point to preferential utilization of TSH by splenic professional APCs and minimal utilization by splenic lymphocytes.

A subset of lymph node T cells express high levels of the TSHr

Expression of the TSHr was studied by flow cytometric analyses on lymph node-derived cells in a manner similar to that described for spleen cells. As shown in Fig. 5A, 52% of lymph node dendritic cells overall expressed the TSHr, further indicating that the presence of the TSH receptor is a feature of most dendritic cells in peripheral lymphoid tissues. A fascinating finding to emerge from these experiments, however, was the observation that unlike T cells in the spleen, a significant proportion of lymph node T cells including both CD4⁺8⁻ and CD4⁻8⁺ cells expressed the TSHr at high levels (Fig. 5, B and C). The proportional expression of TSHr expression on lymph node B cells (Fig. 5, D–F) was similar to that for splenic B cells; binding of TSH to lymph node T cells was blocked with unlabeled recombinant TSH (data not shown).

The presence of the TSHr on lymph node T cells was unequivocally demonstrated by staining for TCRαβ and CD3 in conjunction with TSHr and in experiments in which cells were treated with anti-TSHr Ab with or without complement treatment. As seen in Fig. 6, A and B, ~25% of TCRαβ⁺ and CD3⁺ lymph node lymphocytes coexpressed the TSHr. Cells reacted with anti-TSHr Ab in the absence of complement retained normal staining patterns (Fig. 6, C and E), indicating that binding of anti-TSHr Ab to cells

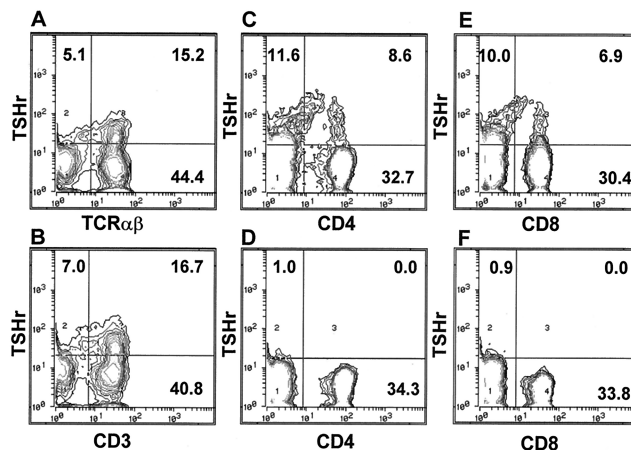


FIGURE 6. Flow cytometric analyses of TSHr and TCRαβ expression (A) and TSHr and CD3 expression (B) on lymph node T cells. Expression of TSHr and CD4 or CD8 on lymph node T cells after treatment with anti-TSHr Ab in the absence of complement (C and E) and the presence of complement (D and F).

did not block the reactivity of biotinylated TSH. However, treatment of Ab-treated cells with complement eliminated TSHr⁺ lymph node cells but had no effect on other T cells (Fig. 6, D and F). These findings, therefore, identify a substantial difference in peripheral T cells based on anatomical compartmentalization in lymphoid tissues.

TSHr⁺ lymph node T cells reside within a population of resting nonmemory cells and are not present until late in development

Three-color flow cytometric analyses were done to determine whether expression of the TSHr on lymph node T cells was associated with a specific lymphocyte subset based on the state of differentiation or according to properties which define functional characteristics. Lymph node cells were stained for TSHr expression in conjunction with CD45RB and CD4 and CD8. CD4⁺ and CD8⁺ T cells each consisted of two populations: a CD45RB^{high} subset that is considered to represent a population of naive nonmemory T cells, and a CD45RB^{low} subset believed to reflect memory T cells. As seen in Fig. 7, TSHr⁺ cells were almost exclusively associated with the CD45RB^{high} subset; few CD45RB^{low} cells were TSHr⁺.

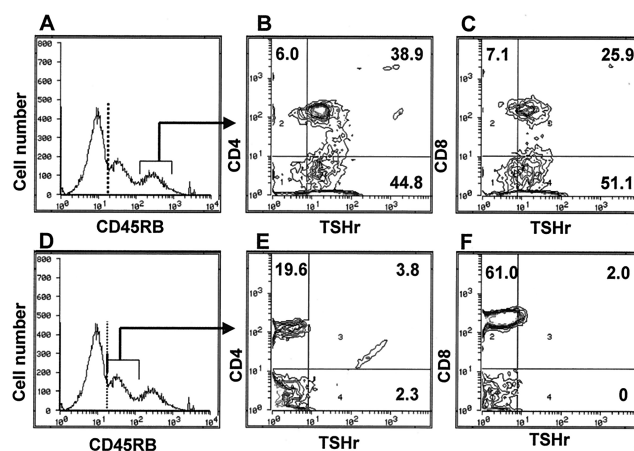


FIGURE 7. Three-color staining for expression of TSHr on CD45RB^{high} and CD45RB^{low} T cells. Note that TSHr⁺ T cells are exclusively expressed on the population of CD45RB^{high} nonmemory T cells.

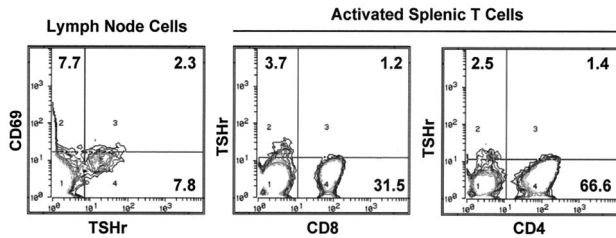


FIGURE 8. CD69 and TSHr expression on freshly isolated lymph node cells and on splenic T cells 48 h postactivation with anti-CD3 Ab, indicating that TSHr⁺ cells belong to a nonactivated T cell subset and that the TSHr is not acquired on T cells following activation via the TCR-CD3 complex.

Under normal conditions, a small proportion of lymph node T cells are activated cells based on CD69 expression. To determine whether the TSHr⁺ cells are newly activated cells, lymph node lymphocytes were stained for expression of TSHr and CD69. As seen in Fig. 8, although ~8% of the cells were CD69⁺, the TSHr was expressed primarily on the CD69⁻ fraction. This finding was then further explored in *in vitro* activation experiments. Splenic T cells, because of their inherent low levels of TSHr expression, were cultured with anti-CD3 mAb for 48 h; viable cells were collected by centrifugation over Ficoll-Paque and stained for TSHr expression. As seen in Fig. 8, neither CD4⁺ nor CD8⁺ T cell lymphoblasts expressed the TSHr after activation. These findings collectively indicate that TSH-responsive T cells reside within a subset of nonmemory cells and that the TSHr in peripheral lymphoid tissues is not acquired during activation of cells.

To gain information about the expression of the TSHr during development, dendritic cells were recovered from the spleens of 18-day-old fetal mice (no T cells were present at that time), and dendritic cells and lymphocytes were obtained from 7-day-old neonatal mice. Interestingly, unlike the phenotypic profile of dendritic cells from adult mouse spleen, the enriched dendritic cell fraction from fetal mice consisted primarily of CD11b⁺ cells, with few CD11c⁺ cells (Fig. 9). Among those cells, few expressed the TSHr. By day 7 postbirth, there was a slight increase in CD11c⁺ cells; however, the proportion of TSHr⁺ cells remained low (Fig. 9). The number of T cells in the spleen at day 7 made up only about 4% of the total cells; few of those cells expressed the TSHr. No T cells were recovered from lymph nodes at that time (data not

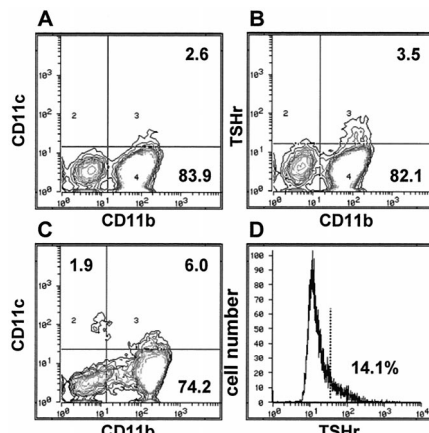


FIGURE 9. Expression of CD11c and CD11b on dendritic cells from the spleen of day 18 fetal mice (A) and day 7 neonatal mice (C). Note the lower percentage of TSHr expression on cells at that time (B and C) compared with adult mice (Figs. 4 and 5).

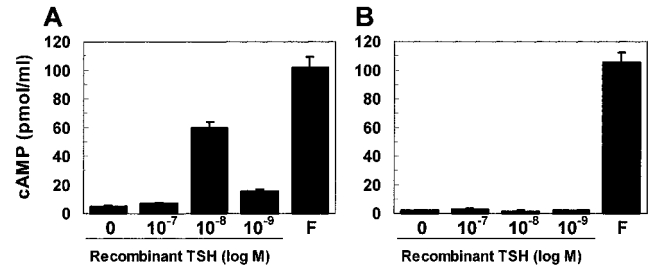


FIGURE 10. Intracellular cAMP levels in freshly isolated splenic dendritic cells from C57BL/6 mice and C.RFTshr^{Jyt/Jyt} mice cultured for 15 min in the absence of TSH, with 10⁻⁷–10⁻⁹ M TSH or 10⁻⁷ M forskolin. Data are mean values ± range for two experiments.

shown). These findings coupled with those described above thus indicate that the TSHr is not associated with immunological maturation during the fetal or neonatal period, further implying a role on mature hematopoietic cells.

TSH enhances the phagocytic activity and IL-1β and IL-12 cytokine activities of dendritic cells

The TSHr is a G protein-coupled receptor which when stimulated by TSH results in rapid increases in intracellular cAMP. To determine whether stimulation of the TSHr on dendritic cells results in a cAMP response, freshly isolated dendritic cells from C57BL/6 mice were cultured with titrated amounts of recombinant TSH, or with forskolin, a known inducer of cAMP activity. C.RFTshr^{Jyt/Jyt} mice, which are incapable of delivering a transmembrane signal due to a point mutation in the TSHr (30), were used as source of control dendritic cells. Note that the pattern of TSHr expression on immune cells from C.RFTshr^{Jyt/Jyt} mice is similar to that observed for normal animals (data not shown). Shown in Fig. 10A, after 15 min of stimulation with TSH, intracellular cAMP levels in dendritic cells from normal mice were elevated significantly over unstimulated cultures in a dose-dependent manner and was approximately two-thirds of that induced by forskolin, thus confirming that the TSHr on murine hematopoietic cells are functionally active. Stimulation of dendritic cells from C.RFTshr^{Jyt/Jyt} mice, by comparison, failed to generate a cAMP signal (Fig. 10B).

Experiments were done to determine how TSH may be involved in the peripheral immune response of dendritic cells. As APCs, dendritic cells are known for their phagocytic ability and for their ability to elaborate cytokines used by other cells of the immune system. Thus, freshly isolated dendritic cells were cultured in the absence or presence of 10⁻⁸ M recombinant TSH (31) with FITC-labeled latex beads to visualize phagocytic activity. Cells were collected after 3 and 9 h and analyzed by flow cytometry for bead uptake. As shown in Fig. 11, at both time intervals there was an increase in the percentage of phagocytic cells when cultured with TSH compared with nonstimulated cultures, with the most pronounced effect occurring within 3 h of culture. Because at the time of analyses, an equivalent number of cells was recovered from both culture groups (data not shown), and because of the short time of culture, the increase in phagocytosis by TSH-treated dendritic cells was not due to expansion of a phagocytic cell population but appears to reflect functional changes of existing dendritic cells. In these experiments, no detectable changes were observed in the expression of TSHr on dendritic cells after activation (data not shown), implying that TSH is involved in the early response of dendritic cells to Ag.

Four dendritic cell cytokines (IL-1β, IL-6, IL-12, and TNF-α) were measured from supernatants after overnight culture of cells with zymosan, a known inducer of phagocytic activity (24), in the

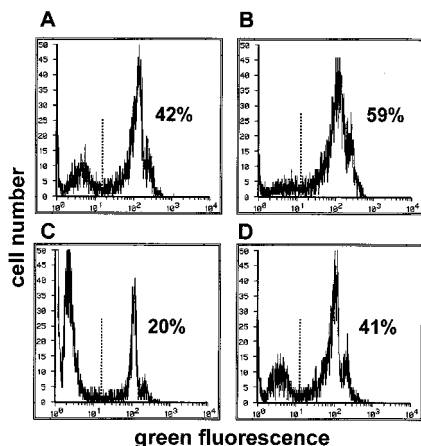


FIGURE 11. FITC-labeled latex bead uptake by untreated splenic dendritic cells (A and C) and TSH-treated dendritic cells (B and D) at 3 h (A and B) or 9 h (C and D) of in vitro culture.

presence or absence of 10^{-8} M recombinant TSH. Findings from these experiments were particularly noteworthy in that coculture of dendritic cells with TSH resulted in significant increases in cytokine activities for IL-1 β (221 vs 143 pg/ml) and IL-12 (19,600 vs 13,200 pg/ml), but had no immunomodulating effects on IL-6 or TNF- α (Fig. 12). Culture of dendritic cells with TSH in the absence of zymosan did not induce cytokine synthesis, indicating that TSH functions primarily as a costimulatory signal during dendritic cell activation. These findings collectively demonstrate 1) that TSH strengthens the overall phagocytic response of dendritic cells and 2) that it leads to a selective increase in the production of two important early acting immune system cytokines.

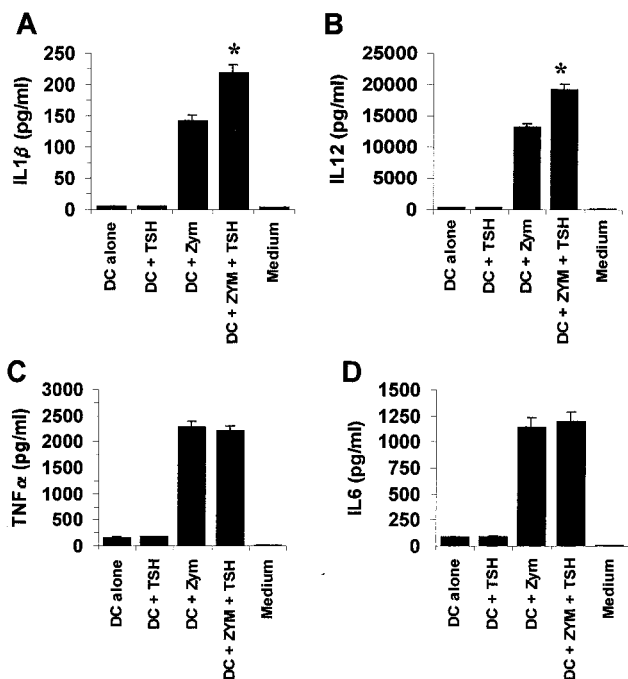


FIGURE 12. Cytokine responses for IL-1 β (A), IL-12 (B), TNF- α (C), and IL-6 (D) from 18-h zymosan-stimulated splenic dendritic cell cultures. Data are mean values \pm SEM of two experiments; *, statistically significant difference ($p < 0.05$) using Student's t test for unpaired observations.

Discussion

The findings reported here provide new information about the pathways linking immune-endocrine communication by HPT hormones within peripheral lymphoid tissues. Although the TSHr has been demonstrated to be expressed on some cells of the immune system as determined from hormone-binding experiments (14, 18, 32–35), the present study compares the presence of the TSHr on murine lymphoid cells by immunoprecipitation and by multicolor flow cytometry to precisely characterize subpopulations of TSH-responsive hematopoietic cells.

The observation of a strong association of TSHr expression with splenic and lymph node dendritic cells is of interest given the important role of those cells in immunity as seen by their ability to influence T cell and B cell activation, to elaborate cytokines used in the inflammatory response, and to serve as a bridge between the adaptive and the innate immune systems (36). This places a large proportion of the TSH-responsive cells of the immune system centrally within the activation/regulation process. In general, the TSHr $^{+}$ dendritic cells identified here conform phenotypically to the myeloid dendritic cell subset based on expression of MHC class II and coexpression of CD11c and CD11b (25, 26, 37, 38). However, a number of additional dendritic cell markers, DEC-205, 33D1, CD8 α , *c-kit*, SCA-2, BP-1, and heat-stable Ag, now have been identified. Although information is incomplete regarding the extent to which those markers define discrete dendritic cell subsets, nonetheless differential expression of those markers has been linked to tissue-associated dendritic cell populations and to developmental differences of dendritic cells defined according to myeloid or lymphoid lineages (37–41). Within peripheral lymphoid tissues, DEC-205 appears to be expressed on most lymph node dendritic cells, but is present on only about half of the total splenic dendritic cells (37, 38). Conversely, CD8 α distinguishes two splenic dendritic cell populations, whereas CD8 α is expressed on a minor component of lymph node dendritic cells. Although in the present study the TSHr was expressed on a high proportion on both the splenic and lymph node dendritic cells, this was by no means a universal feature given that about 30–50% of the cells did not express the TSHr, thus indicating that the TSHr may serve to further delineate and characterize peripheral dendritic cell subpopulations.

Despite rapidly accumulating information about the phenotypic nature of dendritic cells, considerably less is known about the functional differences which exist between the various dendritic cell subsets. In general, however, professional APCs, including dendritic cells, are known to be important for Ag presentation and T cell priming, especially for the activation of naive T cells. For example, compared with the activation requirements of memory and effector T cells, the involvement of dendritic cells during naive T cell activation is particularly critical in terms of the time needed for optimal stimulation. This has been demonstrated in in vitro experiments in which effector T cells can be activated within hours of exposure to Ag by APCs, whereas naive T cells require a minimum of 12 h and possibly up to 30 h (42). The findings reported here of enhanced phagocytic activity of TSH-stimulated dendritic cells places TSH within the very earliest aspects of the immune response. Because resting dendritic cells are considerably more phagocytic than activated dendritic cells (43), a finding also borne-out in the present study by the greater degree of phagocytosis at 3 h vs 9 h after activation (Fig. 11), the effect of TSH appears to be either to prolong the initial state of phagocytosis by dendritic cells or to promote that response in a subset of otherwise nonphagocytic cells. The involvement of TSH in the regulation of dendritic cell cytokine secretion is likewise noteworthy. For example, IL-1 is

critically involved in many aspects of both innate and adaptive immunity as seen by its ability to induce fever (44) and to serve as a costimulatory signal for T cells (45). Similarly, IL-12 synergistically enhances the production of other cytokines, in particular IFN- γ , a T_H1 cytokine with immune modulating (46) and antiviral and antibacterial activities (47).

Although the extent to which TSH participates in the immune response of TSHr⁺ lymph node T cells is not fully evident, TSH has been shown to influence the cellular composition and functional activities of cells of the immune system in a number of ways. C.RFTSHr^{h_{yt}/h_{yt}} mice, which produce but are unable to utilize TSH (30), have increased numbers of peripheral CD4⁺ T cells; this appears to be due to low numbers of CD8⁺ developing thymocytes (48). Interestingly, those animals also have skewed distributions of thymus-derived CD8⁺ T cell subsets in the gut epithelium (18). This suggests that CD8⁺ T cells are particularly susceptible to the effects of TSH, although additional studies will be needed to understand the significance of this in the overall context of immunity.

In mice, hormones, including TSH, have been shown to enhance cytokine responses of hematopoietic cells (31, 49) to increase the cytotoxic activity of NK cells (50) and to serve as a costimulatory factors for mitogen or IL-2-induced T cell proliferation (50). However, because in the latter experiments TSH resulted in increases in proliferation of only about 25–50%, it appears that the costimulatory effects are directed to a subset of the total T cell population, most likely the TSHr⁺ cells described here for the lymph nodes and to a lesser extent the spleen. This also would be consistent with the awareness that signaling by costimulatory molecules such as CD28, as well as the CD4 and CD8 coreceptors, are more critical for optimal activation of naive T cells than for memory cells (51–53). In that vein also, it is interesting that distinct patterns of cytokine production exist among T cells as a function of CD45RB expression, as shown in a study demonstrating that T_H2 cytokines were produced principally by CD45RB^{low} cells, whereas T_H1 cytokines were produced by CD45RB^{high} cells in response to parasitic infections (54). Experiments are currently underway to examine the functional differences between TSHr⁺ T cells in normal C.RF mice and radiation chimeras made from nonmutant C.RF mice reconstituted with bone marrow stem cells from C.RFTshr^{h_{yt}/h_{yt}} mice.

The possibility exists that the primary source of TSH for the immune system is not the pituitary but that it is produced by cells of the immune system itself or by cells intimately associated with lymphoid tissues. Mechanisms for this have been proposed (55) and evidence for this has been demonstrated in the intestinal immune system (18) and in studies showing that TSH can be produced by human monocytic cells (14). The extent to which TRH is also involved in the regulation of this response has not been unequivocally determined; however, the TRH precursor peptide (TRH-Gly) has been reported within the spleen (56). In fact, of 14 non-neural tissues examined in the latter study, the small intestine duodenum and the spleen ranked second and third highest in terms of the concentration of TRH-Gly per milligram of tissue weight (56). This indicates that under normal conditions metabolically active TRH can be converted from existing stores of posttranslational TRH-Gly precursors, thus establishing a mechanism for the rapid secretion of TRH. Still to be understood in this scenario is the precise cell population(s) involved in TRH production, and the nature of the inductive signal for TRH-Gly conversion to TRH; i.e., whether it is mediated by external Ag-driven stimuli or whether signals from the host “feed in” during the early phase of the immune-endocrine process. Some evidence for an internal signal currently exists from experiments which demonstrate increased TRH-Gly levels following thyroid hormone (T₃ or T₄) stimulation

(57). Additional studies aimed at delineating the participation of TRH and other peptide mediators can be done using the hormone-responsive cells described here to understand the functional involvement of immune-endocrine interactions on the immune response.

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