Functional Toll-Like Receptor 4 Conferring Lipopolysaccharide Responsiveness Is Expressed in Thyroid Cells

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Lipopolysaccharide (LPS), a glycolipid found in the cell wall of Gram-negative bacteria, exerts pleiotropic biological effects in different cell types. LPS is mainly recognized by the Toll-like receptor (TLR) 4/MD2/Cluster of differentiation 14 complex (CD14). We previously demonstrated that LPS produced a direct action on thyroid cells, including up-regulation of thyroglobulin gene expression. This work aimed to study further the effect of LPS on thyroid function and to elucidate the mechanism by which LPS is recognized by the thyroid cell. We could detect the transcript and protein expression of TLR4, MD2, and CD14 in thyroid cells, and that these proteins are localized at the plasma membrane. The sodium iodide symporter (NIS) is the transporter involved in the iodide uptake, the first step in thyroid hormonogenesis. We demonstrated that LPS increases the TSH-induced iodide uptake and NIS protein expression. The LPS agonist lipid A reproduced LPS effect, whereas the LPS antagonist, polymyxin B, abrogated it. By the use of anti-TLR4 blocking antibodies and the transient expression of TLR4 dominant-negative forms, we evidenced the involvement of TLR4 in the LPS action. The enrichment of TLR4 expressing Fisher rat thyroid cell line-5 (FRTL-5) cells confirmed that TLR4 confers LPS responsiveness to thyroid cells. In conclusion, we revealed for the first time that all the components of the LPS receptor complex are expressed in thyroid cells. Evidence that the effects of LPS on rodent thyroid function involve TLR4-induced signaling was obtained. The fact that thyroid cells are able to recognize and respond to LPS supports a role of the endotoxin as a potential modifier of thyroid function. **(*Endocrinology* 150: 500–508, 2009)**

Toll-like receptors (TLRs) comprise a family of membrane proteins related to the IL-1 receptor with multiple ligands and a range of signal transduction pathways. At least 11 members of this family suggested to play a significant role in innate immunity have been identified in humans (1). TLRs respond to invading microorganisms by recognizing pathogen-associated molecular patterns of their products. Among the TLR members, TLR4 has been well characterized for its capability to recognize the bacterial endotoxin lipopolysaccharide (LPS), an integral component of the outer surface of all Gram-negative bacteria (2).

Mammals, including humans, are in permanent contact with Gram-negative bacteria and their endotoxins (3). Exposure to circulating LPS occurs from bacterial lysis or division during infectious processes, as well as absorption from gastrointestinal tract during acute physiological stress and trauma (4). LPS exhibits a variety of biological effects mainly acting on immune cells such as macrophages and dendritic cells (5). It is a potent activator of the immune system and inflammatory response. High amounts of the endotoxin in blood lead to systemic changes known as septic shock (6). However, a growing number of LPS actions have been described in other sort of cells, including endothelial (7), epithelial (8), and endocrine cells (9–11).

TLR4 recognizes LPS through a complex mechanism involving several accessory molecules such as Cluster of differentiation 14 (CD14) and MD2. These elements are all important not only for the presentation of LPS and its recognition by TLR4 but also for TLR4 targeting and signaling. CD14 is a high-affinity LPS binding glycoprotein that plays an important role in the LPS action by loading

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Abbreviations: CD14, Cluster of differentiation 14; FITC, fluorescein isothiocyanate; FRTL, Fisher rat thyroid cell line; LPS, lipopolysaccharide; NIS, sodium iodide symporter; TLR, Toll-like receptor.

LPS to the receptor and amplifying its response (2). MD2 is expressed at the cell surface, forming a complex with the extracellular domain of TLR4, and it is indispensable for LPS recognition by TLR4 and cell surface targeting of TLR4. LPS interacts with the receptor TLR4/MD2, and induces oligomerization of TLR4, leading to activation of downstream signaling pathways (12).

TLRs are type I transmembrane glycoproteins with an intracellular region containing a Toll/IL-1 receptor homology domain. This domain interacts with several adaptor molecules activating a cascade of events resulting in transcription factor induction and gene expression (13). Deletions lacking the Toll/IL-1 receptor homology domain as well as a single substitution of proline for histidine at position 712 within the cytoplasmic signaling domain of TLR4 confer LPS hyporesponsiveness (14).

The iodide-concentrating mechanism in the thyroid gland is of considerable physiological importance as the crucial first step in the thyroid hormone biosynthesis (15). Active transport of iodide into the thyroid follicular cells is mediated by the sodium iodide symporter (NIS), an integral plasma membrane glycoprotein. Thyroid-specific differentiated functions as iodide uptake and NIS gene expression are predominantly under the regulation of TSH (15).

The relation of LPS with the thyroid gland has not been widely studied. Our group has recently reported that LPS up-regulates the TSH-stimulated thyroglobulin gene expression, revealing the ability of LPS to modify thyroid-specific gene expression (9). The aim of this study was to elucidate the elements implicated in LPS recognition and their involvement in the effects exerted by LPS on the thyroid cell. Here, we provide strong evidence of the TLR4 expression and its function as the LPS receptor in the Fisher rat thyroid cell line (FRTL)-5 thyroid cell. The CD14 and MD2 proteins were also detected. LPS induced the interaction of TLR4 with CD14, an early event in TLR4 mediated LPS signal, thus suggesting that thyroid cells express a functional LPS receptor. Furthermore, we show that LPS increased the TSH-induced iodide uptake and NIS protein expression by TLR4 mediation. Together, these results support that LPS is able to affect thyroid function under TSH stimulation by involving TLR4-mediated specific recognition.

Materials and Methods

Reagents and antibodies

Phenol extracted LPS from *Escherichia coli* 055:B5 containing less than 1% protein, Monophosphoryl lipid A (Rd mutant) from *E. coli* F583, polymyxin B sulfate, and LPS linked to fluorescein isothiocyanate (FITC) (FITC-LPS) from *E. coli* 055:B5 were from Sigma-Aldrich Corp. (St. Louis, MO). Goat polyclonal antimouse TLR4 antibody and rabbit polyclonal antihuman CD14 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-MD2 antibody was from Abcam, Inc. (Cambridge, MA). Affinity purified site-directed antirat NIS antibodies were a gift from Dr. N. Carrasco (Albert Einstein College of Medicine, Bronx, NY). The monoclonal antibodies against β-actin and FLAG epitope were from Sigma-Aldrich. Polyclonal antimouse α1 subunit of Na+K+ATPase was from Upstate Biotechnology Inc. (Lake Placid, NY).

Cell culture

FRTL-5 and PCCL3 rat thyroid cell lines were grown in DMEM/Ham F-12 medium, supplemented with 5% calf serum (PAA Laboratories GmbH, Pasching, Austria), 1 mIU/ml bovine TSH (a generous gift of the National Institute of Diabetes and Digestive and Kidney Diseases National Pituitary Hormone Program and Dr. A. F. Parlow, National Institutes of Health, Torrance, CA), 10 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 2 μmol/ml glutamine, antibiotics, and antimycotic (Sigma-Aldrich). Cells were maintained for 5–7 d in medium with the same composition but without TSH and containing 0.2% calf serum (basal or starvation medium) before the experiments. TSH-starved (basal) cells were treated with the indicated concentrations of LPS or lipid A in the absence or presence of 0.5 mIU/ml TSH for different periods of time. In experiments in which polymyxin B effect was assayed, antibiotics and antimycotic were removed from the media. After treatments, cell viability was determined using a Trypan blue dye exclusion assay; in all cases cell viability was higher than 97%.

RNA extraction and RT-PCR

Total RNA purification, cDNA synthesis, and PCR were performed as described (16). The primer sequences for TLR4 (555 bp) were 5′-CGCTTTCAGCTTTGCCTTCATTA C-3′ (sense) and 5′-TGCTACTTCCTTGTGCCC TGTGAG-3′ (antisense), for CD14 (364 bp) were 5′-GCTCCCACTCTCAGAATC-3′ (sense) and 5′-CGCAGGGTTCCGAATAGA-3′ (antisense), and for MD2 (379 bp) were 5′-CTTTTCGACGCTGCTTTCTC-3′ (sense) and 5′-ATCTGTGATGGCCCTTAGGA-3′ (antisense). Genomic DNA contamination was excluded because extra bands were not observed, although primer pairs span introns. As a negative control, PCRs where cDNA was replaced by water were performed in parallel. The specificity was confirmed by automatic sequencing. PCR products were separated on 2% agarose gel and visualized with ethidium bromide.

Western blot analysis

Whole protein extracts were prepared as described (9). Proteins (10 μg for NIS, and 50 μg for TLR4, CD14, MD2, and FLAG-tagged TLR4 mutants) were resolved by SDS-PAGE. Blots were incubated with 0.4 μg/ml anti-NIS, 2 μg/ml anti-TLR4, 2 μg/ml anti-CD14 or 2 μg/ml anti-MD2 antibodies in 5% skimmed milk, 0.1% Tween 20 Tris-buffered saline overnight at 4 C. Equal loading was assessed by stripping and probing the same blot with 0.4 μg/ml anti-β-actin or 1 μg/ml anti-α_1-Na+K+ATPase antibodies. Band intensities were measured densitometrically using the ImageJ Image Software (National Institutes of Health, Bethesda, MD).

Flow cytometry and cell sorting

Cells were fixed in 2% paraformaldehyde and incubated for 1 h at room temperature with 5 μg/ml anti-TLR4, 5 μg/ml anti-CD14, or 4 μg/ml anti-MD2 antibodies. Stained cells (10,000–20,000 per sample) were analyzed with a Cytoron Absolute Flow Cytometer (Ortho Diagnostic System, Raritan, NJ). Negative control was obtained by avoiding primary antibody. For LPS binding, cells in culture were incubated with different concentrations of LPS-FITC for 1 h. Cells were fixed in 2% paraformaldehyde for 30 min and analyzed by flow cytometry as described previously. Data were analyzed with WinMDI software (Scripps Research Institute, La Jolla, CA).

For the cell sorting process, cells were not fixed before antibody incubations. All the procedures were performed under sterile conditions. Cells were incubated for 2 h at room temperature with 5 μg/ml anti-TLR4 antibody. Negative control was done by omitting primary antibody. Positive events were recovered using a BD FACS Aria Flow Cytometer (BD Biosciences, San Jose, CA). After sorting, the recovered cells were centrifuged and resuspended in growing media and cultured as described previously.

Immunoprecipitation

Cells were lysed in 1 ml lysis buffer containing 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, and protease inhibitors. Five hundred micrograms of total protein from each sample were precleared for 20 min at 4 C with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology), and immunoprecipitated with 4 μg anti-TLR4 and control goat IgG or 2 μg of anti-CD14 and control rabbit IgG antibodies overnight at 4 C under constant rotation. Immune complexes were allowed to bind to Protein A/G PLUS-Agarose during 4 h at 4 C under

constant rotation; agarose beads were washed with lysis buffer, resuspended in sample buffer, and boiled. Aliquots (40 μl) of supernatants were used for Western blot analysis as described previously.

Immunohistochemistry

Normal rat thyroids were fixed with 10% buffered formalin during 24 h before paraffin embedding for histological examination and immunohistochemistry. The heat-induced antigen retrieval method was performed in 10 mM sodium citrate (pH 6.0) buffer at 95 C. Endogenous peroxidase activity was quenched in 3% H_2O_2 in methanol. Tissue sections were blocked with 5% horse serum, followed by overnight incubation at 4 C in a humid chamber with 5 μg/ml anti-TLR4, anti-CD14, or anti-MD2. Immunostaining was detected using labeled streptavidin-biotin reagents (L.V. Dako LSAB+ Kit/HRP; Dako Corp., Carpinteria, CA) and 3,3′-diaminobenzidine (Sigma-Aldrich) as chromogenic substrate. Tissue sections were counterstained with hematoxylin and mounted with 1,3-diethyl-8-phenylxanthine. Slices were analyzed by light microscopy at ×400 and ×1000 magnification (Nikon Eclipse TE2000-U; Nikon Corp., Tokyo, Japan). For negative control the primary antibody was replaced with 5 μg/ml purified IgG (Santa Cruz Biotechnology) from the same species.

Iodide uptake

Cells were incubated with buffered Hanks' balanced salt solution [140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.55 mM glucose, and 10 mM HEPES (pH 7.5)] containing 20 μM KI supplemented with 10 μCi/μl carrier-free $Na^{125}I$ for 45 min at 37 C. The amount of ^{125}Iodide accumulated in the cells was extracted with 95% ice-cold ethanol and then quantified in a γ-counter. The DNA amount was determined by the diphenylamine method on the material not extracted by ethanol after trichloroacetic acid precipitation (16). Iodide uptake was expressed as picomol of iodide per μg DNA (pmol/μg DNA).

Transient transfection and cDNA constructs

When FRTL-5 cells cultured in six-well plates reached 50–60% confluence, growing media were changed by basal media as described previously. After 3–4 d cells were transiently transfected by Lipofectamine Plus reagent (Invitrogen Corp., Carlsbad, CA) as specified by the manufacturer. Treatments of cells were initiated after 48 h transfection. Constructs containing the mouse TLR4 FLAG-tagged dominant-negative mutant TLR4(P712H) and the human TLR4 FLAG-tagged dominant-negative mutant with a deletion in the intracellular domain TLR4(ΔCt) were a gift from Dr. E. A. Kurt-Jones (University of Massachusetts Medical School, Worcester, MA) and Dr. M. Smith (University of Virginia, Charlottesville, VA), respectively.

Statistical analysis

Analysis of multiple intergroup differences was conducted by one-way ANOVA. As posttest, the Student-Newman-Keuls multiple comparisons test was used. Comparisons between two groups were made using the Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

Expression of TLR4 and accessory molecules (CD14 and MD2) in the thyroid cell

TLR4 receptor is accepted to be the main mediator of the LPS response (2). The presence of the TLR4 mRNA transcript was detected by RT-PCR in samples derived from starved FRTL-5 and PCCL3 thyroid cell lines (Fig. 1A). The TLR4 mRNA expression was also detected in whole rat thyroid tissue. We studied the mRNA expression of the accessory molecules, CD14 and MD2. The mRNA expression of these molecules was present in FRTL-5 and PCCL3 rat thyroid cell lines, as well as in the normal rat thyroid tissue (Fig. 1A).

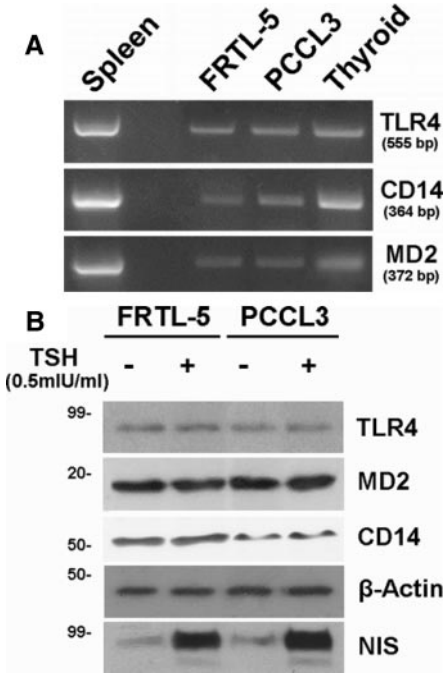

FIG. 1. Expression of TLR4 and the accessory molecules (CD14 and MD2) in thyroid cells. Basal FRTL-5 and PCCL3 thyroid cells and whole rat thyroid tissue were assayed for expression of TLR4 and accessory molecules at mRNA and protein levels. A, Representative RT-PCR for TLR4, CD14, and MD2. Total RNA was extracted from starved FRTL-5 and PCCL3 thyroid cell lines and whole rat thyroid tissue. Spleen was run in parallel as a positive control. B, Representative Western blot of whole proteins obtained from FRTL-5 and PCCL3 cells at basal state and after TSH treatment (0.5 mIU/ml, 48 h). The same blots were reprobed with an anti-β-actin antibody to assess equal loading. In the *lowest panel*, NIS expression is shown to ensure effective TSH response of the cells.

The expression of TLR4, CD14, and MD2 at the protein level was analyzed by Western blot. TLR4 and the accessory molecules were found to be expressed in both thyroid cell lines (Fig. 1B). A possible role of TSH in the regulation of the proteins involved in LPS recognition was investigated by analyzing the protein expression in TSH-treated cells for 48 h. No difference in the expression of TLR4 or any accessory molecule at the protein level was observed in cells under TSH treatment compared with basal cells (Fig. 1B). The well-characterized up-regulation of NIS in response to TSH treatment (15) was used as a positive control to ascertain TSH responsiveness of the cells.

TLR4 and accessory molecules are localized at the plasma membrane in the thyroid cell

TLR4 has been located at the plasma membrane in most of the cell types (17), although a functional intracellular expression has been described in certain sorts of cells (18, 19). To analyze the subcellular localization of the LPS recognition molecules in FRTL-5 thyroid cells, we performed different approaches. By flow cytometry, we found a positive staining for TLR4, CD14, and MD2 in nonpermeabilized basal cells (Fig. 2A), supporting their expression at the plasma membrane. When flow cytometry analysis of TLR4, CD14, and MD2 was performed in TSH-

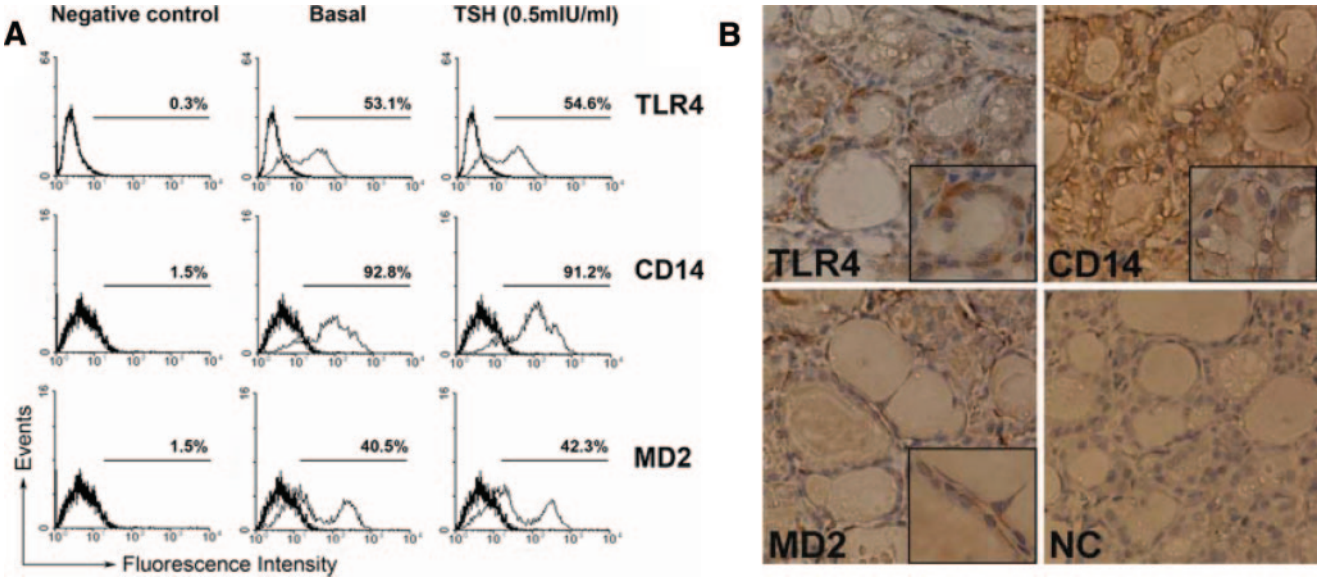

FIG. 2. TLR4 and accessory molecules are localized at the plasma membrane in the thyroid cell. A, Nonpermeabilized FRTL-5 cells were analyzed for TLR4, CD14, and MD2 expression by flow cytometry under basal or TSH (0.5 mIU/ml, 48 h) conditions. Negative control represents the samples only labeled with the secondary FITC-conjugated antibody. The histogram in each panel plots the number of cells (events) on the vertical axis against the fluorescence intensity of the labeled antibody bound to the indicated protein on the horizontal axis. The *horizontal line* on each histogram indicates the fluorescence range that contains the indicated percentage of positive cells for the assayed protein in the population. Each histogram is representative of three independent experiments. B, Immunohistochemical staining for the LPS-receptor TLR4 and the accessory molecules CD14 and MD2 in normal whole rat thyroid tissue. Evident expression with a basolateral localization in the follicular cells is observed for the three molecules analyzed (magnification, ×400). *Insets* display a particular region of the same figure (magnification, ×1000). Negative control (NC) shows the absence of nondesired staining when specific antibodies were replaced by a nonrelated IgG from the same species.

treated (0.5 mIU/ml for 48 h) and nontreated cells, no differences were observed in their expression levels or distribution patterns (Fig. 2A). In cell surface biotinylation experiments, we observed the presence of TLR4, CD14, and MD2 in the biotinylated fraction, corroborating that these molecules are present in the plasma membrane (supplemental data 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The expression of these proteins at the plasma membrane was also confirmed by immunofluorescence analysis. We detected a well-defined plasma membrane localization pattern of these proteins. A colocalization between TLR4 and CD14, as well as TLR4 and MD2 was demonstrated (supplemental data 1).

Immunohistochemistry assay in slices from normal rat thyroid tissue was used to confirm the protein expression and subcellular localization of TLR4, CD14, and MD2. Despite the expected heterogeneity of the thyroid tissue, a clear immunoreactivity was detected in the thyroid follicular cells. The immunopositivity was present mainly in the basolateral surface and in part associated with the basolateral membrane of follicular cells, but not in other thyroid cell types (Fig. 2B). Membrane-associated NIS protein staining in follicular cells was used as a positive control (supplemental data 1).

LPS binds to the plasma membrane of thyroid cells and induces receptor accessory protein interactions

The binding of the endotoxin to the thyroid cell was analyzed using FITC-LPS as probe. Basal FRTL-5 cells were incubated with increasing concentrations of FITC-LPS (0.01–10 μg/ml). We found that LPS bound to nonpermeabilized cells by a saturable process, indicating the ability of LPS to bind specifically to the plasma membrane of the thyroid cell (Fig. 3A). To corroborate the binding specificity, FITC-LPS was competed with an excess of unlabeled LPS (500×). In this condition an almost complete reduction of fluorescence staining was observed (Fig. 3A). When cells were incubated with FITC-LPS in the presence of an anti-TLR4 blocking antibody, a significant, although partial, reduction of FITC staining was observed. This result evidenced that TLR4 is involved in the LPS recognition and binding to the plasma membrane.

To assess the possible formation of a functional receptor complex upon LPS stimulation, we performed coimmunoprecipitation experiments. Starved FRTL-5 cells were treated or not with LPS (100 ng/ml), TSH (0.5 mIU/ml), or LPS plus TSH during 30 min. After treatment, cell lysates were immunoprecipitated with either control goat IgG or anti-TLR4 antibody. The coprecipitated proteins were examined for the presence of CD14 and MD2 by Western blot. As shown in Fig. 3B, in the presence of LPS, a strong coprecipitation of TLR4-CD14 occurs, whereas the TLR4-MD2 complex remains constant along the treatments. No coprecipitation took place when goat IgG was used instead of anti-TLR4 antibody (data not shown). To confirm further this finding, the same cell lysates were immunoprecipitated with anti-CD14 antibody, and coprecipitated TLR4 was detected by Western blot. Induction of the CD14-TLR4 interaction was also observed under LPS treatment (data not shown).

LPS acts on thyroid cell function, increasing the TSH-stimulated iodide uptake and NIS protein expression

To investigate the action of LPS on thyroid cell function, we analyzed its effect on iodide uptake and NIS expression, two

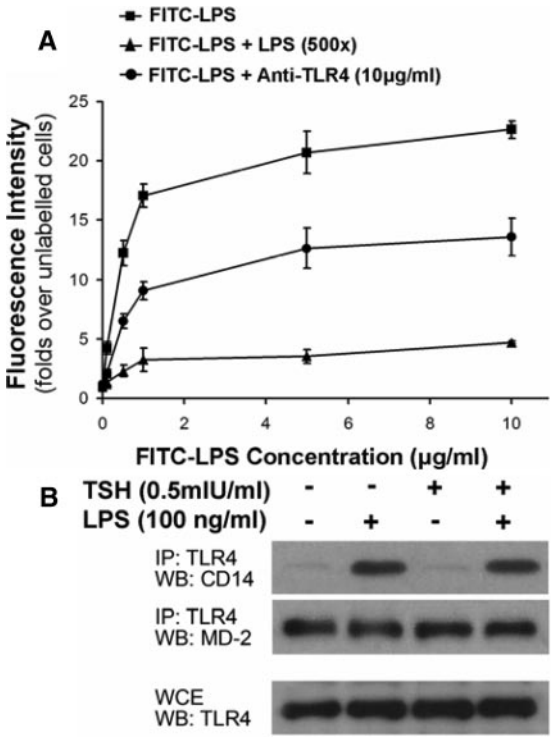

FIG. 3. LPS binds to the thyroid cell plasma membrane. A, Nonpermeabilized basal FRTL-5 cells were assayed for LPS binding using FITC-LPS as probe. Cells were incubated for 1 h with 0.01–10 μg/ml probe (■), probe plus an excess (×500) of unlabeled LPS (▲), or probe plus 10 μg/ml anti-TLR4 blocking antibody (●), and analyzed by flow cytometry. Results were normalized in relation to the fluorescence intensity of unlabeled cells and expressed as mean ± SD of three independent experiments. B, Lysates from TSH-starved FRTL-5 cells treated or untreated as indicated for 30 min were immunoprecipitated (IP) with either control goat IgG or anti-TLR4 antibody. The bound proteins were detected by Western blot with anti-CD14 or anti-MD2 antibodies. *Lower panel*, Whole cell extracts (WCE) were also subjected directly to Western blot analysis with the same antibody used for immunoprecipitation to show that equal amounts of TLR4 were expressed at the starting point.

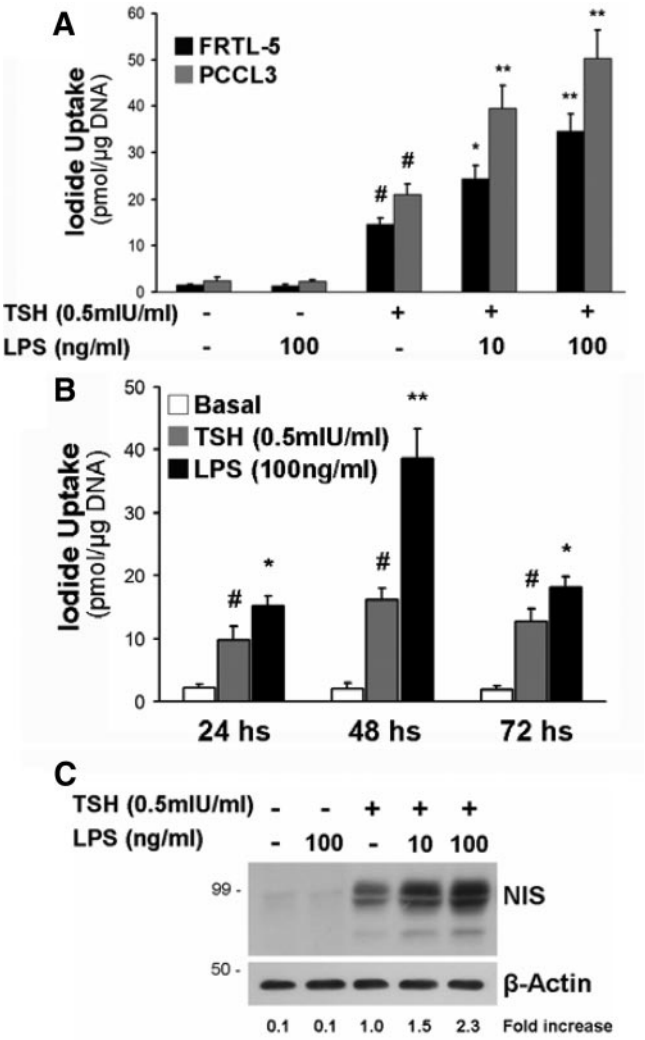

FIG. 4. LPS increases TSH-stimulated iodide uptake and NIS protein expression in thyroid cells. Basal FRTL-5 and PCCL3 cells were treated with LPS alone (100 ng/ml), TSH alone (0.5 mIU/ml), or TSH plus LPS (10 and 100 ng/ml) for 48 h. A, Iodide uptake level in basal, LPS, TSH, and TSH plus LPS treated cells. FRTL-5 cells are shown in *black bars* and PCCL3 in *gray bars*. Each value represents the mean ± SD of pmol I^-/μg DNA of three independent experiments done in triplicate. *, $P < 0.01$ and **, $P < 0.001$ *vs.* TSH alone; #, $P < 0.005$ *vs.* basal cells (Student-Newman-Keuls multiple comparisons test). B, LPS-induced increase in the TSH-stimulated iodide uptake varies with the time of incubation. Basal FRTL-5 cells were treated as previously described for different times (24–72 h). Each value represents the mean ± SD of pmol I^-/μg DNA of three independent experiments done in triplicate. *, $P < 0.05$ and **, $P < 0.001$ *vs.* TSH alone; #, $P < 0.001$ *vs.* basal cells (Student-Newman-Keuls multiple comparisons test). C, Representative Western blot analysis of whole cell extract assayed for NIS expression. β-Actin, a nonrelated thyroid housekeeping gene, was used to correct loading differences. Densitometric analysis was performed to determine the relative increase (fold increase) of NIS normalized to β-actin. The value of TSH alone was set up arbitrarily as 1.0. The results represent the mean of three independent experiments.

well-defined markers of thyroid differentiation (20). Basal FRTL-5 and PCCL3 cells were treated with LPS (10 and 100 ng/ml) in the presence or absence of TSH (0.5 mIU/ml) for 48 h. LPS was able to increase the iodide uptake level in TSH-treated cells. The stimulation was dose dependent (Fig. 4A). Incubation with LPS alone did not modify iodide uptake in comparison with that of basal cells. A similar pattern of LPS stimulation was observed in both thyroid cell lines (Fig. 4A).

TSH augmented iodide uptake at all time periods (24–72 h) with a slightly maximal effect at 48 h (Fig. 4B). The presence of LPS stimulated the TSH-induced iodide uptake level with a maximal increase after 48 h treatment. At 72 h there was an important diminution in the response to LPS, even though the LPS-induced stimulation of iodide uptake was still significant when compared with that of TSH alone (Fig. 4B).

To study the effect of LPS on the NIS protein expression, basal FRTL-5 cells were treated as described for iodide uptake assay for 48 h. An increase in the NIS protein level was observed under treatment with LPS in the presence of TSH (Fig. 4C). As observed in iodide uptake experiments, LPS alone was not able to induce NIS biosynthesis.

The stimulatory action of LPS on the thyroid cell function is mediated by TLR4

To obtain insights into the specificity of LPS effect and the TLR4 functionality, we performed treatments with agonists and antagonists of LPS action in FRTL-5 cells. The purified active domain of LPS, lipid A (100 ng/ml), known as a specific TLR4-agonist (21), was able to mimic the LPS-induced stimulation of iodide uptake (Fig. 5A) and NIS protein expression (Fig. 5B).

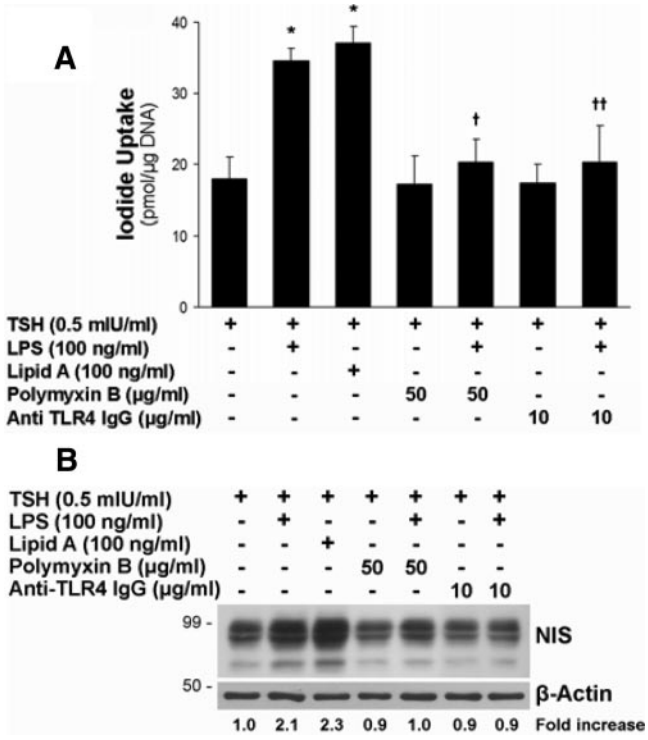

FIG. 5. TLR4 mediates the stimulatory action of LPS. A, Iodide uptake levels from basal FRTL-5 cells treated with TSH (0.5 mIU/ml), TSH plus LPS (100 ng/ml), or lipid A (100 ng/ml), TSH and TSH plus LPS in the presence of polymyxin B (50 μg/ml), or an anti-TLR4 blocking antibody (10 μg/ml) for 48 h. Polymyxin B and anti-TLR4 antibody were added to the cell culture medium 1 h before and during the treatment. Results are expressed as mean ± SD of pmol I^-/μg DNA of three independent experiments done in triplicate. *, $P < 0.001$ *vs.* TSH alone; †, $P < 0.001$ and ††, $P < 0.005$ *vs.* TSH plus LPS (Student-Newman-Keuls multiple comparisons test). B, Representative Western blot analysis of NIS expression from whole proteins extracted from FRTL-5 cells treated as described previously. β-Actin was used as loading control. Densitometric analysis was performed to determine the relative increase (fold increase) of NIS normalized to β-actin. The value of TSH alone was set up arbitrarily as 1.0. The results represent the mean of three independent experiments.

Preincubation of cells for 1 h with the LPS-antagonist polymyxin B (50 μg/ml) (22) produced a blockage of the LPS stimulatory effect on iodide uptake (Fig. 5A) and NIS protein expression (Fig. 5B). Polymyxin B was able to abolish LPS effect but did not affect the stimulation exerted by TSH on the analyzed parameters.

We analyzed the LPS action in the presence of a functional TLR4-specific blocking antibody. Preincubation of cells with the anti-TLR4 antibody (10 μg/ml) resulted in a blockage of the LPS-induced stimulation of iodide uptake (Fig. 5A). Similar blockage by the TLR4 antibody was observed on the LPS-induced NIS protein expression (Fig. 5B). When cells were treated with a nonrelated goat IgG, the effect of LPS was unaffected (data not shown). Treatment with the anti-TLR4 antibody did not affect TSH-induced iodide uptake or NIS protein expression.

Functional blockage of endogenous TLR4 function by dominant-negative mutants abolishes the LPS effect

The involvement of the TLR4 signaling in LPS-induced stimulatory response in the thyroid cell was corroborated by blockage of the endogenous TLR4 function after overexpression of TLR4 dominant-negative mutants. FRTL-5 cells were transiently transfected with constructs codifying the nonsignaling dominant-negative TLR4 mutants TLR4(P712H) or TLR4(ΔCt). As shown in Fig. 6A, the exogenous expression of each of these mutants led to a significant inhibition of the LPS-stimulated iodide uptake. Similarly, the overexpression of the mutants produced a blockage of the LPS effect on NIS protein expression (Fig. 6B). No changes in the TSH-stimulated iodide uptake and NIS expression were observed in the presence of the mutants.

The dominant-negative TLR4 mutants contain a FLAG tag in the N terminal. By using a monoclonal anti-FLAG antibody, we corroborated the expression of the mutants in the transfected cells by Western blot (Fig. 6B, *lower panel*). The adequate targeting of the mutant TLR4(ΔCt) to the plasma membrane was monitored by flow cytometry in nonpermeabilized transfected cells (supplemental data 2).

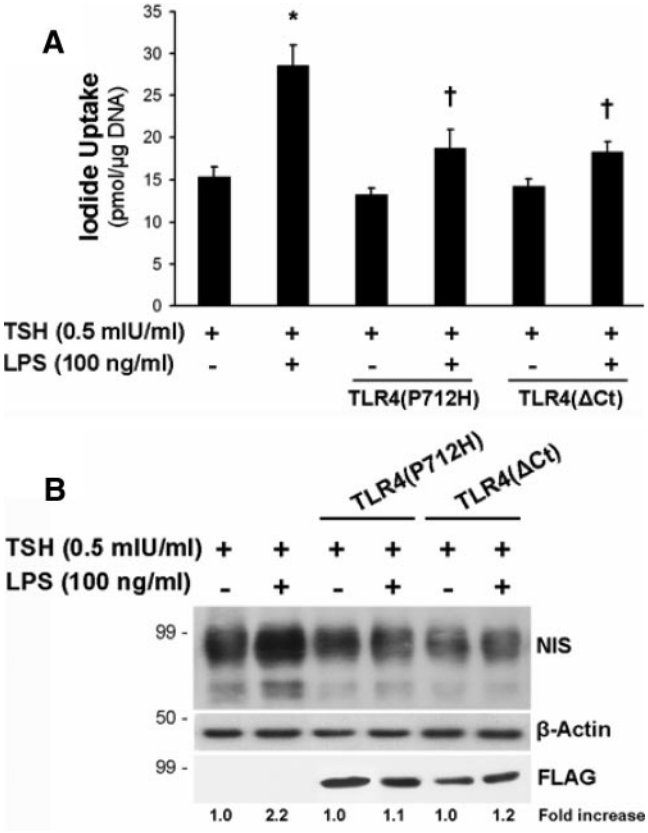

FIG. 6. Functional blockage of endogenous TLR4 function by dominant-negative mutants abolishes the LPS effect. A, Iodide uptake analysis in basal FRTL-5 cells nontransfected or transfected (0.5 μg DNA/24-well) with the TLR4 nonsignaling constructs [TLR4(P712H) or TLR4(ΔCt)] treated with TSH (0.5 mIU/ml) or TSH plus LPS (100 ng/ml). Results are expressed as mean ± SD of pmol I^-/μg DNA of two independent experiments done in triplicate. *, $P < 0.001$ *vs.* TSH alone; †, $P < 0.005$ *vs.* TSH plus LPS (Student-Newman-Keuls multiple comparisons test). B, Representative Western blot showing that transfection of FRTL-5 with the TLR4 dominant-negative mutants (2 μg DNA/6-well) results in a blockage of LPS stimulus on NIS expression. β-Actin staining was used to control loading and FLAG staining to demonstrate the expression of the TLR4 mutants in transfected cells. Densitometric analysis was performed to determine the relative increase (fold increase) of NIS normalized with β-actin. Values of TSH alone in each individual condition were set up arbitrarily as 1.0. The results represent the mean of two independent experiments.

Enrichment of the FRTL-5 population in TLR4-positive cells leads to a higher response to LPS and lipid A

The FRTL-5 cell line is heterogeneous just as thyroid follicular cells (23, 24). Therefore, whereas TLR4 was revealed to be localized at the plasma membrane of FRTL-5 cells, not all the cells expressed this receptor. We detected TLR4 and MD2 expression in only 30–50% of the cells; meanwhile, CD14 was expressed in as many as 90% (Fig. 2A).

We took advantage of this dual population of FRTL-5 cells for TLR4 expression to confirm further the involvement of this receptor in the response to LPS. Using cell sorting, FRTL-5 cells were enriched in the TLR4-positive population. Cell sorting enrichment was confirmed by flow cytometry analysis of the native and sorted populations that showed a high enrichment in TLR4-positive cells. TLR4 protein expression was significantly increased in TLR4-sorted cells (supplemental data 3). The TLR4-enriched cell population was treated with TSH (0.5 mIU/ml) in the presence or absence of LPS (100 ng/ml) or lipid A (100 ng/ml). Under treatment with both agents, we found an increase in iodide uptake levels in the TLR4-sorted cells compared with that observed in the native FRTL-5 population, without differences in the response to TSH (Fig. 7A). When NIS protein analysis was performed, a higher expression of the protein was induced by LPS in the TLR4-enriched cells compared with the original population in the presence of TSH (Fig. 7B).

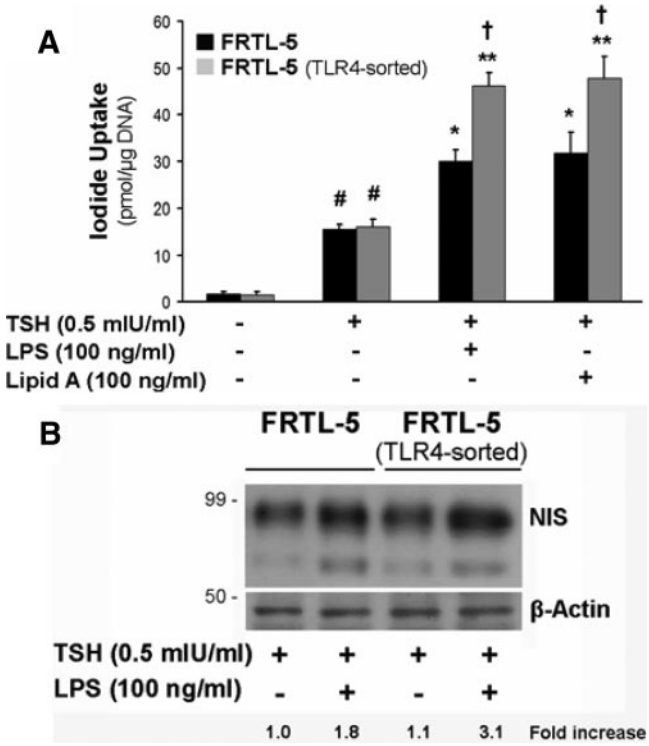

FIG. 7. Enrichment of the FRTL-5 population in TLR4-positive cells leads to a higher response to LPS and lipid A. FRTL-5 population was enriched in TLR4-positive cells. A, Iodide uptake levels from native or TLR4-sorted FRTL-5 cells starved or under TSH (0.5 mIU/ml) stimulation with or without LPS (100 ng/ml) and lipid A treatment. Each value represents the mean ± SD of pmol $I^-/\mu g$ DNA of three independent experiments done in triplicate. #, $P < 0.01$ *vs.* basal state; *, $P < 0.01$ and **, $P < 0.001$ *vs.* TSH alone; †, $P < 0.01$ *vs.* TSH plus LPS in nonsorted FRTL-5 cells (Student-Newman-Keuls multiple comparisons test). B, Representative Western blot of whole cell proteins extracted from starved native FRTL-5 or TLR4-sorted cells treated with TSH (0.5 mIU/ml) in the presence or absence of LPS. β-Actin shows equal protein loading along different conditions. Densitometric analysis was performed to determine the relative increase (fold increase) of NIS normalized with β-actin. The value of TSH alone in native FRTL-5 was set up arbitrarily as 1.0. The results represent the mean of three independent experiments.

Discussion

In the present paper, we revealed for the first time the presence of the LPS receptor TLR4 and its localization at the plasma membrane, as well as TLR4 involvement as a functional mediator of LPS action in the thyroid cells. These findings are in accordance with previous observations indicating the expression of TLR mRNAs in human thyroid follicles (25, 26). Although TLR4 has been mainly involved in the immune response, a growing number of reports have described the existence of TLR4 in nonimmune cells. Thus, TLR4 expression has been demonstrated in some endocrine cells such as those of the adrenal gland (11), pancreas (10), and the enteroendocrine component of intestine (27). Concordantly, it has been proposed that a TLR4-mediated response to LPS could be involved in immune-adrenal relationships (11), and that TLR4-expressing enteroendocrine cells could have a role in intestinal homeostasis and immunosuppression (27). Our results then strengthen the interesting hypothesis raised by these studies about the contribution of bacterial interactions with nonimmune cells to the regulation of immune response and inflammation.

It has been very well demonstrated that TLR4 is the main mediator of the LPS action in several cell types (1, 12, 28). However, cells expressing TLR4 but not the accessory molecules CD14 or MD2 have been found not to be LPS responsive (29, 30). Here, we evidenced the concomitant expression of TLR4 and the accessory molecules that indicate the availability of the elements of the LPS receptor complex in FRTL-5 thyroid cells. Our observations on the presence of TLR4, CD14, and MD2 in the rat whole thyroid tissue support that the expression of these proteins in FRTL-5 and PCCL3 is not a particular characteristic of these cell lines. Because the mRNA expression of TLR4 and accessory molecules in the whole rat thyroid tissue could be originated from other cell types such as mesenchymal, endothelial, or immune cells, we proved by immunohistochemistry the actual presence of these molecules at the follicular thyroid cell.

This study reports the ability of LPS to stimulate iodide uptake and the expression of the iodide transporter, NIS in TSH-stimulated thyroid cells. In accordance, our previous observations indicated that LPS increased TSH-induced thyroglobulin expression in FRTL-5 cells (9). On the contrary, it has been reported that LPS had no significant effect on the TSH-induced iodide uptake and hormone synthesis in cultured human thyroid follicles from Graves' disease tissues (25). This discrepancy could be explained by the use of dissimilar experimental conditions or possible differences between the two species. The stimulation of NIS expression by LPS demonstrated here reinforces the influence of bacterial endotoxins on thyroid-specific gene expression previously proposed by our group (9). Interestingly, it has been demonstrated that LPS is able to up-regulate diverse functions in other endocrine cells in *in vivo* and *in vitro* models. Thus, LPS

increased GH expression in rat adenohypophysial cells and GH secretion *in vivo* (31), as well as cortisol secretion in human adrenocortical cells (11). In pancreatic β-cells, LPS was able to affect insulin secretion but not synthesis in a glucose-dependent manner (10). These findings allow speculation that the LPS released during the infectious process would be able to produce functional modifications on endocrine cells, including the thyroid cell.

The absence of changes in the TLR4 expression and subcellular localization under TSH stimulation observed here seems to indicate a constitutional expression of this receptor. Similar considerations could be made for the accessory molecules. However, here we showed that LPS lacked the ability to stimulate iodide uptake or NIS expression in TSH-starved cells. These data suggest a cross talk between the signal activated by TSH, which strongly controls the expression of genes involved in thyroid hormonogenesis (15), and the TLR4-dependent signal triggered by LPS, which is able to enhance further thyroid-specific gene expression. Whatever the mechanism involved, the ability of LPS to act on TSH-treated cells is in favor that the endotoxin could modify the thyroid cell function under physiological conditions of TSH stimulation.

Here, we show that LPS is able to bind to the cell surface of the thyroid cell. The LPS binding was demonstrated to be highly specific by competition experiments. We found a LPS-inducible interaction of TLR4 with CD14 and a constant interaction of TLR4 with MD2 that supports the induction of a functional LPS-receptor complex in the thyroid cell. These findings are in agreement with previous reports indicating that LPS is able to induce a TLR4-CD14 complex, whereas an already preformed TLR4-MD2 complex is present at the plasma membrane in several cell types (32–34). The presence of an anti-TLR4 blocking antibody reduced the LPS binding, indicating the involvement of TLR4 in the interaction of LPS with the thyroid cell.

The present observations favor the hypothesis that the LPS-induced changes in the thyroid cell are mediated by functional TLR4 receptors. The specificity of the LPS action was evidenced by the fact that lipid A was able to reproduce the LPS-induced stimulation of iodide uptake and NIS expression, and that the LPS action on these parameters was abolished by polymyxin B. The functional activity of TLR4 was demonstrated by the blockage of LPS-stimulated iodide uptake and NIS expression in the presence of a specific neutralizing anti-TLR4 antibody. Different dominant-negative TLR4 mutants have been widely used to study the role of TLR4 in the cell response to LPS (35, 36). We confirmed the TLR4 involvement by the abolishment of the LPS stimulatory action on the two thyroid parameters when each of two dominant-negative mutants of TLR4 was transiently expressed in thyroid cells. Moreover, the functional mediation of the TLR4 receptor was reinforced by our observation that the LPS-induced increase of iodide uptake and NIS expression in a TLR4-enriched population was higher than that found in native FRTL-5 cells.

The ability of the FRTL-5 thyroid cell to express the LPS receptor complex reported here raises the question of a possible direct action of LPS on thyroid function. Because the presence of LPS *in vivo* induces profound modifications, including those of the immune response and pituitary-thyroid axis (37, 38), it is difficult to establish the actual impact of the endotoxin on the thyroid function. Environmental agents such as toxins and viruses have been proposed to be involved in some thyroid diseases (39). Thus, it has been suggested that the thyroid dysfunction induced during viral infection could lead to the development of thyroid autoimmunity (25). Analogously, bacterial agents have been implicated in the induction of autoimmune thyroid disease (39, 40). In agreement, it has been reported that the endotoxin plays a role as an environmental factor in some diseases involving autoantibodies or autoantigen-specific T cells (41, 42). It is intriguing to know whether effects of LPS similar to those demonstrated here in FRTL-5 could take place under certain conditions in human thyroid cells. Although it may be premature to speculate that the presence of TLR4 could contribute to make thyrocytes susceptible to be affected during bacterial infection, the expression of the LPS recognition complex places the follicular cells at the junction of the innate and adaptive immune response (37, 43, 44), a postulated checkpoint in the development of autoimmune diseases (45, 46).

In conclusion, these results reveal a novel functional expression of the LPS receptor, TLR4, and the accessory molecules CD14 and MD2 at the cell surface of the thyroid follicular cells. We describe for the first time the ability of LPS to up-regulate TSH-stimulated iodide uptake and NIS expression in thyrocytes. Evidence for the ability of FRTL-5 cells to recognize and respond to LPS is provided. Further studies in other cell models, including human thyroid cells, will certainly contribute to elucidating a possible role of this pathway in thyroid disease.

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