Nuclear Factor (NF)-κB-dependent Thyroid Hormone Receptor β1 Expression Controls Dendritic Cell Function via Akt Signaling*§

Iván D. Mascalfroni1, María del Mar Montesinos2, Vanina A. Alamino1, Sebastián Susperreguy1, Juan P. Nicola1, Juan M. Ilarregui5, Ana M. Masini-Repiso5, Gabriel A. Rabinovich5,8, and Claudia G. Pellizas1,4

From the 1Centro de Investigaciones en Bioquímica Clínica e Inmunología, Consejo Nacional de Investigaciones Científicas y Técnicas, Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, 5000 Córdoba, the 2Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, C1428 Ciudad de Buenos Aires, and the 4Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428 Ciudad de Buenos Aires, Argentina

Despite considerable progress in our understanding of the interplay between immune and endocrine systems, the role of thyroid hormones and their receptors in the control of adaptive immunity is still uncertain. Here, we investigated the role of thyroid hormone receptor (TR) β1 signaling in modulating dendritic cell (DC) physiology and the intracellular mechanisms underlying these immunoregulatory effects. Exposure of DCs to triiodothyronine (T3) resulted in a rapid and sustained increase in Akt phosphorylation independently of phosphatidylinositol 3-kinase activation, which was essential for supporting T3-induced DC maturation and interleukin (IL)-12 production. This effect was dependent on intact TRβ1 signaling as small interfering RNA-mediated silencing of TRβ1 expression prevented T3-induced DC maturation and IL-12 secretion as well as Akt activation and IκB-ε degradation. In turn, T3 up-regulated TRβ1 expression through mechanisms involving NF-κB, suggesting an autocrine regulatory loop to control hormone-dependent TRβ1 signaling. These findings were confirmed by chromatin immunoprecipitation analysis, which disclosed a new functional NF-κB consensus site in the promoter region of the TRβ1 gene. Thus, a T3-induced NF-κB-dependent mechanism controls TRβ1 expression, which in turn signals DCs to promote maturation and function via an Akt-dependent but PI3K-independent pathway. These results underscore a novel unrecognized target that regulates DC maturation and function with critical implications in immunopathology at the cross-roads of the immune-endocrine circuits.

Received for publication, September 29, 2009, and in revised form, December 14, 2009 Published, JBC Papers in Press, December 17, 2009, DOI 10.1074/jbc.M109.071241

The endocrine and immune systems are interconnected via a bidirectional network in which hormones affect immune function, and, in turn, immune responses are reflected in neuroendocrine changes. This bidirectional communication is possible as both systems share common ligands (hormones and cytokines) and their specific receptors (1). Thyroid hormones (TH)5 play critical roles in differentiation, growth, and metabolism. The classic genomic actions of TH are mediated by nuclear TH receptors (TR) that act mainly as hormone-inducible transcription factors. Several TRα and TRβ isoforms are encoded by the TRA and TRB genes, respectively. The TRα1, TRα2, TRβ1, and TRβ3 isoforms are widely expressed, whereas TRβ2 is predominantly restricted to the hypothalamus-pituitary axis (2). Recent emerging evidence has also characterized the interactions of TR with co-repressors proteins, namely the nuclear co-repressor and the silencing mediator of retinoid and TH receptors. These effects involve histone deacetylase activity that mediates TR silencing in the absence of triiodothyronine (T3) and several co-activator proteins that exhibit histone acetylation activity in the presence of this hormone (2). However, the notion of classical or genomic mechanisms as unique actions mediated by TRs has been challenged in the past decade by descriptions of TH actions that involve extranuclear (nongenomic) effects in a variety of cell types. These TH-dependent pathways are associated to extranuclear TR localized within the cytoplasm and the plasma membrane (3–5) and to TR-dependent effects mediated by the cell surface αvβ3 integrin (6). Several cytoplasmic T3 actions mediated by TR are linked to activation of the PI3K pathway in alveolar cells (7) and human fibroblasts (8). Moreover, activation of Akt, a critical component of cell growth and survival (9), has been detected in pancreatic islet β cells upon engagement of TRβ1 and activation of P13K-p85 (10).

Despite considerable progress in understanding the interplay between distinct hormones and the immune cell network, the

* This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas Grant PIP-5325 (to C. G. P.), Agencia Nacional de Promoción Ciencia y Técnica Grants PICT 2005-33139 (to A. M. M.) and 2006-603 (to G. A. R.), and grants from Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (to C. G. P.), Cancer Research Institute (to G. A. R.), and Fundación Sales (to G. A. R.).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5, “Methods,” and additional references.

1 Research Fellow of Consejo Nacional de Investigaciones Científicas y Técnicas.

2 Research Fellow of Fondo para la Investigación Científica y Tecnológica.

3 Member of the Research Career of Consejo Nacional de Investigaciones Científicas y Técnicas.

4 To whom correspondence should be addressed: Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000, Córdoba, Argentina. Fax: 54-351-4333048; E-mail: claudia@fcq.unc.edu.ar.

5 The abbreviations used are: TH, thyroid hormone; TR, thyroid hormone receptor; DC, dendritic cell; T3, triiodothyronine; PI3K, phosphatidylinositol 3-kinase; IL, interleukin; siRNA, small interfering RNA; IDC, immature DC; LPS, lipopolysaccharide (endotoxin); PBS, phosphate-buffered saline; PE, phycoerythrin; CHX, cycloheximide; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MHC, major histocompatibility complex; ChIP, chromatin immunoprecipitation; JNK, c-Jun N-terminal kinase.
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role of TH in the control of immune cell physiology has received scarce attention with studies almost exclusively focused on effector B and T lymphocytes (11, 12). However, the role of TR signaling in the initiation of adaptive immunity remains elusive.

Dendritic cells (DCs) are highly specialized antigen-presenting cells that recognize, process, and present antigens to naive T cells for the induction of antigen-specific immune responses (13). Given the remarkable plasticity of these cells, manipulation of their function to favor the induction of DCs with immunogenic or tolerogenic properties could be exploited to stimulate or attenuate immune responses (14). After in vitro exposure to lipopolysaccharides (LPS) or other microbial products, DCs undergo activation and maturation through different signaling pathways, including MAPKK1/ERK, which favors DC survival, and the Akt and NF-κB pathways, which allow for DC maturation (15, 16). Signaling through NF-κB also determines the increased expression of major histocompatibility complex (MHC) II and co-stimulatory molecules, release of proinflammatory cytokines and chemokines, and DC migration and recruitment. This coordinated process leads to sustained T cell activation, and the regulation of DC physiology with critical implications in immunopathology.

EXPERIMENTAL PROCEDURES

Mice—Female C57BL/6 mice (B6; H-2b) were obtained from Ezeiza Atomic Center (Buenos Aires, Argentina). Mice were maintained under specific pathogen-free conditions and used at 6–10 weeks old. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and the local institutional animal care committee.

DC Preparation and Culture—DCs were obtained as described by Inaba et al. (18). Briefly, bone marrow progenitors were collected from the femurs of C57BL/6 mice, cultured in RPMI 1640 medium, 10% fetal calf serum depleted of TH by Ezeiza Atomic Center (Buenos Aires, Argentina). Mice were cultured in complete medium (1.5 × 10⁶ cells/300 μl) in the presence of T3 (5 nm) or LPS (100 ng/ml) and 10 μM LY294002 hydrochloride, 50 nM wortmannin, 20 μM H89, or 3 mM CHX added only once, at the beginning of individual experiments, for different time periods (0–60 min or 18 h) at 37 °C. LY-294002, wortmannin, H89, and CHX were resuspended in stock solution, according to the manufacturer’s instruction, and stored at −20 °C. Control DC cultures were kept under the same conditions as treated cells but in the absence of drugs. The final concentration of DMSO was identical, in every culture, irrespective of individual treatments. The reactions were stopped at the indicated times by adding cold saline solution, and samples were centrifuged. Cell pellets were resuspended in loading buffer (60 mM Tris, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromphenol blue, and 5% (v/v) 2-mercaptoethanol), boiled at 96 °C for 5 min, and stored at −80 °C. Samples were then separated by SDS-PAGE (10 or 12%), transferred to polyvinylidene difluoride membranes (Sigma), and then blocked with 5% (w/v) bovine serum albumin in PBS containing 0.05% (v/v) Tween 20. Membranes were then blotted with anti-phospho-Akt, anti-phospho-ERK 1/2, anti-phospho-p38 MAPK, or anti-phospho-JNK antibodies, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG from Santa Cruz Biotechnology. Anti-TRβ1 (C4) (rabbit polyclonal antibody) was purchased from Rockland. Antibodies directed against phospho-Akt (Ser-473), phospho-ERK 1, and phospho-ERK 2, (Thr-202/Tyr-204, rabbit polyclonal), phospho-p38 MAPK (Thr-180/Tyr-182, rabbit polyclonal), and phospho-JNK (Thr-183/Tyr-185, rabbit polyclonal) were obtained from Cell Signaling. Anti-IκB-α mouse monoclonal antibodies were purchased from Pharmingen. Anti-glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody was obtained from Sigma. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were from Santa Cruz Biotechnology. LY294002 hydrochloride and wortmannin (PI3K inhibitors), H89 (selective cAMP-dependent protein kinase inhibitor), and cycloheximide (CHX) were obtained from Sigma. DCs were cultured in complete medium (1.5 × 10⁶ cells/300 μl) in the presence of T3 (5 nm) or LPS (100 ng/ml) and 10 μM LY294002 hydrochloride, 50 nM wortmannin, 20 μM H89, or 3 mM CHX added only once, at the beginning of individual experiments, for different time periods (0–60 min or 18 h) at 37 °C. LY-294002, wortmannin, H89, and CHX were resuspended in stock solution, according to the manufacturer’s instruction, and stored at −20 °C. Control DC cultures were kept under the same conditions as treated cells but in the absence of drugs. The final concentration of DMSO was identical, in every culture, irrespective of individual treatments. The reactions were stopped at the indicated times by adding cold saline solution, and samples were centrifuged. Cell pellets were resuspended in loading buffer (60 mM Tris, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromphenol blue, and 5% (v/v) 2-mercaptoethanol), boiled at 96 °C for 5 min, and stored at −80 °C. Samples were then separated by SDS-PAGE (10 or 12%), transferred to polyvinylidene difluoride membranes (Sigma), and then blocked with 5% (w/v) bovine serum albumin in PBS containing 0.05% (v/v) Tween 20. Membranes were then blotted with anti-phospho-Akt, anti-phospho-ERK 1/2, anti-phospho-p38, or anti-phospho-JNK antibodies, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG. Specific bands were developed by ECL (Amersham Biosciences). Membranes were stripped and exposed to anti-Akt 1/2/3 (Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology), or anti-α-tubulin (Sigma) monoclonal antibodies.

Immunofluorescence Microscopy—Bone marrow-derived DCs were generated as described above and cultured on coverslips for 3 days. After treatments, cells were fixed in 4% (w/v) paraformaldehyde, permeabilized in 0.25% (v/v) Triton X-100
in PBS, blocked for 1 h in PBS, pH 7.4, plus 3% (w/v) bovine serum albumin fraction V (Fisher), incubated with primary antibodies (mouse anti-TRβ1 sc-738, rabbit anti-Akt 1/2/3 sc-8312, and rabbit anti-PI3K p85α sc-423, Santa Cruz Biotechnology) at a dilution of 1:100 for 1 h, washed, and further incubated with Alexa-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Molecular Probes) for 1 h at a dilution of 1:1000. Nuclei were stained with 4,6-diamidino-2-phenylindole for 5 min, and samples were washed in PBS and mounted on glass slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) for examination using an inverted fluorescence microscope equipped for confocal microscopy (Olympus Flawview FV500, Hamburg, Germany). Images were captured using Openlab 3.1 software (Improvision, Lexington, MA) at a magnification of ×1000. Negative controls, including omission of primary antibodies, were also performed.

**Immunoprecipitation**—Cells were lysed in 1% (v/v) Nonidet P-40, 0.2 mM phenylmethlysulfonyl fluoride, 10 mM sodium fluoride, 0.7 mg/ml pepstatin, and 25 mg/ml aprotinin in 1× PBS. After 10 min on ice, samples were sonicated and centrifuged at 12,000 × g for 15 min. Protein content was measured using the Bradford method (Bio-Rad). Cell lysates (400 μg) were incubated for 2 h with 30 μl of A/G-protein (Santa Cruz Biotechnology). After pre-clearing, cell lysates were incubated overnight at 4 °C with mouse anti-TRβ1 or rabbit anti-Akt 1/2/3 antibodies (1 μg, Santa Cruz Biotechnology) and 30 μl of freshly prepared A/G-protein. The immunoprecipitates were run onto a 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Sigma). Membranes were then blocked with 5% (w/v) nonfat dry milk in 1× PBS, 0.1% Tween 20 (v/v) for 1 h at room temperature and probed with anti-phospho-Akt (1:500) or anti-PI3K-p85α (1:500) rabbit antibodies or an anti-TRβ1 (1:500) mouse antibody, respectively. The membranes were stripped and exposed to a rabbit anti-TRβ1 (C4) (1:500) or a mouse anti-TRβ1 (C4) (1:500) antibodies diluted in 5% (w/v) nonfat dry milk in 1× PBS, 0.1% Tween 20 overnight at 4 °C under gentle rocking. After washing in PBS, 0.1% Tween 20 (v/v), membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at a dilution of 1:4000 in 5% (w/v) milk in PBS, 0.1% Tween 20 (v/v) for 1 h. Immunoreactivity was visualized by using the ECL immunodetection system (Amersham Biosciences) following the manufacturer’s instructions.

**Flow Cytometry**—Cells were washed twice with PBS supplemented with 2% (v/v) fetal calf serum and resuspended in 10% (v/v) fetal calf serum in PBS. Cells were then incubated with the following fluorochrome-conjugated monoclonal antibodies for 30 min at 4 °C: fluorescein isothiocyanate-anti-CD11c, PE-anti-IA/IE (MHC II), PE-anti-CD40, PE-anti-CD80, and PE-anti-CD86 (Pharmingen). To determine TRβ1 expression, DCs were fixed with 1% (w/v) paraformaldehyde, treated with fluorescence-activated cell sorter permeabilizing solution (BD Biosciences), stained with a mouse anti-TRβ1 antibody (sc-738, Santa Cruz Biotechnology) at a dilution of 1:100, and further incubated with an Alexa-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 1 h at a dilution of 1:1000. Intracellular cytokine detection was assessed by flow cytometry as described (20) using PE-conjugated anti-IL-12 monoclonal antibody (from BD Biosciences). Briefly, DCs incubated with T3 or LPS in the absence or presence of 1 μM BAY 11-7082 or 2 μM sulfasalazine (NF-κB inhibitors), 10 μM Akt 1/2 kinase inhibitor (with no inhibitory activity on PI3K) added only once at the beginning of the individual experiments were exposed to brefeldin A (10 μg/ml; Sigma) for the last 6 h of cell culture. Cells were then fixed with 1% (w/v) paraformaldehyde, treated with fluorescence-activated cell sorter permeabilizing solution, and stained with an optimal concentration of anti-cytokine monoclonal antibody or an appropriate isotype control (all from BD Biosciences). Cells (at least 10,000 viable cells) were then analyzed in a FACSArria flow cytometer (BD Biosciences) using Flowjo software (Tree Star, Ashland, OR).

**Cytokine Determination**—IL-12p70 detection was performed in cell culture supernatants using standard capture enzyme-linked immunosorbent assays. Coating antibody included a rat anti-mouse IL-12p70 monoclonal antibody (clone C15.6, Pharmingen). Detection antibody included biotinylated rat anti-mouse IL-12p70 monoclonal antibody (clone C17.8, Pharmingen). Streptavidin-horseradish peroxidase and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma) were used as enzyme and substrate, respectively.

**RNA Interference**—Cells were plated onto six multwell plates and grown in complete medium. After 24 h, cells were transfected with siRNA SmartPool for thyroid hormone receptor β, Akt, Rel A, or PI3K (p85α) (Dharmacon, Lafayette, CO). Dharmacon also provided the negative control siRNA (scramble siRNA). Transfection was conducted as described previously (21). Briefly, 200 pmol of annealed siRNA or scramble siRNAs were incubated with 7 μl of GenePorter (Gene Therapy Systems) in a volume of 100 μl of RPMI 1640 medium (serum-free) for 30 min and added to 900 μl of DC culture (2 × 10⁶ cells) as described above. After 4 h of incubation, an equal volume of RPMI 1640 medium supplemented with 20% (v/v) fetal calf serum was added. After 24–48 h, transfected DCs were washed and used for subsequent experiments. Silencing of target genes was checked by immunoblot analysis.

**Preparation of Nuclear and Cytoplasmic Extracts**—Nuclear and cytoplasmic DC extracts were obtained by subcellular fractionation as described previously (22). The supernatant containing cytoplasm was collected and frozen at −80 °C or used immediately. The nuclear pellet was resuspended in 50 μl of ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and the tube was vigorously rocked at 4 °C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at 4 °C, and the supernatant was frozen in aliquots at −80 °C or used immediately.

**ChIP and PCR Analysis of Precipitated DNA**—Formaldehyde cross-linking, whole cell extract preparation, immunoprecipitation, DNA purification, and PCR analysis were performed as described previously (23) with some modifications (24, 25). Antibodies used for immunoprecipitation include anti-NF-κB p65 (10 μg, sc-8008; Santa Cruz Biotechnology) and an unrelated isotype control (2 μl whole serum). Sonication treatment resulted in average DNA fragment sizes of 0.5–1 kb. PCR (25–35 cycles) was carried out in a 20-μl volume with 1:100,
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Real Time PCR Quantitation of Co-immunoprecipitated Promoter Fragments—The relative proportions of co-immunoprecipitated promoter fragments were determined by real time PCR using the primers listed above. PCR was performed using the iCycler iQ real time PCR detection system (7500 real time PCR system. Applied Biosystems) and a SYBR Green-based kit for quantitative PCR (Bio-Rad). The relative proportions of co-immunoprecipitated promoter fragments were determined based on the threshold cycle (C_T) value for each PCR. Real time PCR data analysis followed the methods described in ChIP-quantitative PCR primer assays, quantitative real time PCR assays for chromatin immunoprecipitation analyses (user manual, SuperArray Bioscience Corp.). For promoter studies, a ∆C_T value was calculated for each sample by subtracting the C_T value for the input (to account for differences in amplification efficiencies and DNA quantities before immunoprecipitation) from the C_T value obtained for the immunoprecipitated sample. A ∆∆C_T value was then calculated by subtracting the ∆C_T value for the T_T-treated sample from the ∆C_T value of the corresponding control sample. Fold changes in occupancy (NF-κB-p65 ChIP relative to control ChIP) were then determined by raising 2 to the -∆∆C_T power. For promoter fragment analysis, each sample was quantified in triplicate on at least two separate occasions and from at least three independent immunoprecipitation experiments. Mean ± S.D. values were determined for each fold difference, and these values were subsequently used in two-tailed paired t tests to determine statistical significance, as reported in the figures. A melt curve analysis was performed for each sample after PCR amplification to ensure that a single product of expected melt curve characteristics was obtained.

Statistical Analysis—Statistical analysis was performed using the Student’s paired t test. Wilcoxon nonparametric test for paired data was used to determine the significance of the time-response curves. p values less than 0.05 were considered statistically significant. To adjust the significance level for multiple comparisons, Bonferroni correction was applied using a corrected significance level of 0.017. All experiments were performed at least in triplicate.

RESULTS

T3 Triggers PI3K-independent Akt Activation in Bone Marrow-derived Mouse DCs—Given the immunostimulatory effects of T3 and the preferential cytoplasmic localization of TRβ1...
on DCs (17), in this study we investigated the intracellular pathways triggered by TRβ₁ signaling on these cells and their relevance in DC physiology. Because the Akt signaling pathway fine-tunes the immunostimulatory function of DCs (26), we first examined the activation of this pathway in T₃-stimulated (DC_T₃) and control (DC_Control) DCs. Bone marrow-derived iDCs were cultured in the presence or in the absence of T₃ (5 nM) for 5, 15, 30, and 60 min and 18 h. A significant increase in
Ser-473 phosphorylation of Akt was detected as early as 5 min following exposure of DCs to T3 with a peak detected at 30 min of incubation (versus control). The increase in Akt phosphorylation persisted even at 18 h of addition of the thyroid hormone (Fig. 1A). Remarkably, T3-induced Akt activation seemed to be independent of PI3K, as pretreatment of iDCs with PI3K inhibitors (wortmannin and LY294002) was not capable of preventing Akt phosphorylation (Fig. 1A). In turn, T3 effects did not require de novo synthesis of proteins because the addition of CHX to iDC cultures did not alter Ser-473 phosphorylation of Akt (Fig. 1A). Notably, Akt phosphorylation was barely observed on DCs exposed to LPS (Fig. 1B).

Given the involvement of other major signaling pathways during DC maturation (27), we further examined the phosphorylation of ERK 1/2, p38, and JNK following treatment with T3 as well as the effect of the inhibition of cAMP-activated protein kinase (protein kinase A (PKA)) signaling on T3-induced Akt phosphorylation. In contrast to LPS, T3 could not trigger ERK 1/2, p38, or JNK phosphorylation when added for different time periods to DC cultures (Fig. 1C). Moreover, H89, a potent inhibitor of PKA, could not prevent T3-induced Akt phosphorylation (Fig. 1D). In addition, as PP2A (protein phosphatase 2A) negatively regulates Akt activity in various systems (28), we further explored whether a reduction of PP2A C levels could underlie T3-mediated up-regulation of Akt. Of note, the levels of PP2A C were not reduced following treatment of DCs with T3. On the contrary, PP2A C was slightly increased compared with DCs cultured with medium alone (supplemental Fig. 1).

Collectively, these results support the concept of an Akt-dependent PI3K-independent mechanism underlying T3 modulation of DC physiology. In

**FIGURE 3. TRβ1 does not co-localize with PI3K-p85 in bone marrow-derived iDC.** Bone marrow-derived iDCs were cultured for 18 h with or without T3 (5 nM) (DC_T3 and DC_Control, respectively). A, immunofluorescence staining. DCs were immunostained for TRβ1 (green fluorescence) and PI3K-p85 (red fluorescence). All images were acquired and analyzed by confocal microscopy as described under “Experimental Procedures.” B, co-immunoprecipitation analysis. Immunoprecipitation (IP) was performed in whole cell lysates using an anti-TRβ1 antibody or an irrelevant isotype control antibody. Western blot (WB) analysis for PI3K (p85α) was performed as described under “Experimental Procedures.” The identities of the bands were corroborated with the input control. Blots are representative of three independent experiments.
addition, this mechanism does not appear to involve other major signaling pathways, including ERK 1/2, p38, JNK, PKA, or PP2A.

**TRβ1 Co-localizes and Interacts with Akt in a Ligand-independent Manner**—Given the pronounced changes induced by T3 in Akt phosphorylation, we further examined the possible interactions between TRβ1 and Akt in DC_{T3} and DC_{Control}. Confocal microscopy and co-immunoprecipitation analyses revealed a striking co-localization of TRβ1 and Akt both in DC_{Control} as well as in DC_{T3} (Fig. 2, A–D). This effect was similar in the absence or presence of T3 and was specific as shown by the lack of signal when we used an irrelevant isotype control antibody for immunoprecipitation (Fig. 2, B–D). This finding suggests that TRβ1 and Akt may interact physically in the cytoplasm of DCs independently of the presence of T3, supporting the notion of nongenomics actions of T3 involving Akt activation. Consistent with the lack of effect of PI3K inhibitors on T3-induced Akt phosphorylation, we could observe no co-localization (Fig. 3A) or physical interaction (Fig. 3B) between TRβ1 and PI3K, neither in the absence nor in the presence of T3, as revealed by confocal microscopy and co-immunoprecipitation.

In several biological systems, Akt is recruited to the plasma membrane where it is activated by phosphorylation. Once activated, pAkt is translocated to different cell compartments, including the nucleus (10). Immunoblot analysis of pAkt in the nuclear fraction of DCs revealed the ability of T3 to trigger rapid recruitment of pAkt (Ser-473) to the nucleus following exposure to T3 (Fig. 4). Moreover, a significant proportion of TRβ1 shuttled to the nuclear fraction (Fig. 2A and Fig. 4), in agreement with TRβ1 cytoplasmic-nuclear translocation described in other cell types (29). Collectively, these observations reinforce the concept of a PI3K-independent Akt activation mediated by T3 in bone marrow-derived iDCs.

**TRβ1 Transduces PI3K-independent Akt- and NF-κB-dependent Signals That Mediate DC Maturation and IL-12 Production**—The contribution of Akt and NF-κB pathways to T3-mediated effects was substantiated by functional experiments. These involved evaluation of cell surface phenotypic markers and IL-12 production on iDCs exposed to T3 or LPS in the presence or absence of Akt or NF-κB inhibitors or following siRNA-mediated silencing of Akt, NF-κB, or PI3K. Similar to DCs matured in the presence of LPS, exposure of DCs to T3 resulted in enhanced percentage of cells expressing the co-stimulatory molecules CD40, CD80 (B7.1), and CD86 (B7.2), as well as enhanced expression (mean fluorescence intensity) of these cell surface molecules compared with DCs cultured in the absence of maturation stimuli (Fig. 5A). Remarkably, this effect was prevented when DCs were matured with either T3 or LPS in the presence of a specific NF-κB inhibitor (BAY 11-7082). However, pharmacological disruption of Akt specifically prevented T3 but not LPS effects (Fig. 5A), suggesting that T3 triggers DC maturation via Akt and NF-κB-mediated mechanisms. The involvement of these pathways was also observed at the level of cytokine production. Exposure to T3 resulted in a substantial increase in the frequency of IL-12-producing CD11c^{+} DCs as well as in the amounts of secreted IL-12p70; these effects were significantly prevented by specific inhibitors of Akt (Akt 1/2 kinase inhibitor; p < 0.01), NF-κB (BAY 11-7082; p < 0.01) (Fig. 5, B and C), or sulfasalazine (data not shown). Remarkably, similar findings were observed when Akt and NF-κB expression was interrupted by specific siRNA (Fig. 6, B and C). Small interfering RNA-mediated silencing of Akt or NF-κB successfully prevented T3-induced DC maturation, as shown by cell surface phenotypic markers (Fig. 6B) and IL-12 production (Fig. 6C). In contrast, T3 effects could not be abrogated when DCs were exposed to this hormone in the presence of ERK 1/2, p38, or JNK inhibitors (data not shown). Moreover, siRNA-mediated silencing of PI3K could not abrogate T3 effects (Fig. 6, B and C). The specificity and efficacy of all siRNAs used are shown in Fig. 6A. Thus, T3 delivers maturation signals to iDCs via selective modulation of Akt (independently of PI3K) and NF-κB.

**Critical Role of TRβ1 Signaling in T3-induced DC Maturation**—To investigate whether TRβ1 is critical in T3-induced DC maturation, we interrupted TRβ1 signaling on these cells by siRNA-mediated silencing strategies. Transfection of TRβ1-siRNA but not scramble siRNA almost completely abrogated TRβ1 expression, as shown by flow cytometric analysis of permeabilized DCs (Fig. 7A) and immunoblot analysis of total cell lysates (Fig. 7B). More importantly, siRNA-mediated TRβ1 silencing substantially prevented T3-induced DC maturation, as shown by a lower percentage of cells expressing MHC II, CD40, CD80, and CD86, and lower expression levels of these cell surface molecules (Fig. 7C). Furthermore, blockade of TRβ1 signaling completely prevented T3-induced IL-12 secretion (Fig. 7D), suggesting a critical function of this hormone receptor in transducing intracellular signals that endow DCs with the ability to polarize Th1 responses. This finding was confirmed with four separate siRNA duplexes (supplemental Fig. 3).

To further examine the involvement of TRβ1 in regulating T3-induced Akt and NF-κB activation, we stimulated control or
TRβ1-siRNA-transfected DCs with T₃ for 30 min or 18 h and analyzed Akt phosphorylation and IκB-ε degradation. Remarkably, TRβ₁ silencing completely abrogated T₃-induced Akt phosphorylation and IκB-ε degradation, although it did not alter basal Akt or IκB-ε expression. (Fig. 8). Likewise, TRβ₁ or Akt silencing prevented T₃-induced IκB-α degradation (supplemental Fig. 4). Taken together, these results imply a critical role of intact TRβ₁ expression in coupling T₃ binding, Akt- and NF-κB-dependent signaling, and DC maturation.

**T₃ Regulates TRβ₁ Expression on DCs via NF-κB**—It is well established that TRβ₁ expression is differentially regulated by T₃ in a tissue-specific manner (30) and that TH responses are dependent not only on TH but also on TR levels (2). Hence, we studied the effects of T₃ on TRβ₁ expression on iDCs. Notably, treatment of iDCs with T₃ (5 nM) for 30 min considerably increased TRβ₁ protein expression, which remained elevated up to 18 h and returned to control levels after 24 h of exposure to T₃ (Fig. 9A) (data not shown). Remarkably, pharmacological inhibition of the NF-κB pathway by BAY 11-7082 as well as blockade of NF-κB expression by siRNA (Rel A) silencing substantially prevented T₃-induced up-regulation of TRβ₁ (Fig. 9A, left and right panels), suggesting involvement of this transcription factor as a regulator of T₃-induced TRβ₁ expression in bone marrow-derived DCs.

The gene encoding mouse TRβ is located in chromosome 14 (31) from 18,493,474 to 18,870,600 bp. Analysis of the promoter region corresponding to the start site of the TRB1 coding sequence (ATG, 18,814,391 bp) up to −1300 bp revealed a putative NF-κB consensus site from −644 to −652 bp (GGATGTCCC). ChIP analysis using PCR probes that amplify a 206-bp fragment of the TRB1 promoter harboring the putative NF-κB consensus site and DNA
obtained from anti-NF-κB (p65) immunoprecipitated chromatin revealed a positive signal (Fig. 9B). Remarkably, this signal was considerably amplified following treatment of iDCs with T3 for 30 min and 18 h (Fig. 9, B and C). Our results identify a functional consensus region for NF-κB in the promoter region of the TRB1 gene, which could be involved in T3-induced NF-κB-dependent regulation of TRB1 expression in bone marrow-derived iDCs. These results were confirmed by functional assays using a luciferase reporter fused to various fragments of the TRB1 promoter (containing or not the NF-κB consensus site) co-transfected together with an NF-κB expression vector in COS-7 cells (supplemental Fig. 5). These findings provide the first evidence of TRB1 as an NF-κB target gene with critical implications in the control of inflammatory responses.

DISCUSSION

DCs are critical “decision-making” cells that must integrate signals from several pathways and receptors, including those arising from engagement of uptake and pattern-recognition receptors, pro-inflammatory and anti-inflammatory cytokines, chemokines, and hormones to determine the type and magnitude of adaptive immune responses (32). Recently, we demonstrated an essential role for T3 in promoting DC maturation and Th1-type cytokine secretion (17). As the T3-TRβ1 interaction may represent an attractive target for rational manipulation of the immunogenicity of DCs (32), here we investigated the molecular mechanisms and signaling pathways underlying these immunostimulatory effects. Collectively, the results presented in this study underscore a key role of TRβ1 signaling in coupling the thyroid system with the initiation of adaptive immunity, through intracellular pathways involving selective activation of Akt and NF-κB. In addition, we identified TRB1 as a novel NF-κB target gene with critical implications in the development of inflammatory responses.

FIGURE 5. T3 promotes DC maturation and IL-12 production via Akt- and NF-κB-dependent pathways. Bone marrow-derived iDCs were treated with T3 (5 nM; DC_T3), LPS (100 ng/ml; DC_LPS), or left untreated (DC_Control) for 18 h in the absence or presence of an NF-κB inhibitor (BAY 11-7082) or Akt 1/2 kinase inhibitor added 30 min before maturation stimuli (A–C) as described under “Experimental Procedures.” At the end of the experimental period, DCs were washed and processed for flow cytometry of cell surface markers (A), intracytoplasmic IL-12 staining (B), and enzyme-linked immunosorbent assay for IL-12p70 (C). A, flow cytometry analysis of phenotypic markers (co-stimulatory molecules) of DCs treated or not with T3 (5 nM) or LPS (100 ng/ml) (black histograms, nonspecific binding determined with isotype-matched control antibodies; white histograms, phenotypic markers). B, intracytoplasmic detection of IL-12p70. Following incubation of DCs with T3 or LPS, cells were incubated with brefeldin A for 4 h, fixed, permeabilized, and stained with an anti-IL-12p70 antibody as described under “Experimental Procedures.” The percentage of IL-12p70 cells was determined by flow cytometry; histograms are gated on CD11c cells. Numbers in plots indicate the percentage of positively stained cells in the gate. Data are from one representative of six independent experiments. C, enzyme-linked immunosorbent assay of IL-12p70 in supernatants of DC_Control, DC_T3, or DC_LPS in the absence or presence of specific inhibitors (*, p < 0.01 versus DC_Control; #, p < 0.01 versus DC_T3; Student’s t test). Data are the means ± S.D. of three independent experiments.
The ability of T₃ to access the cytoplasmic and nuclear compartments and transactivate TH-regulated genes is well established. This action, classically referred to as genomic effect, occurs within hours to days, which is consistent with the typical hormone functions, including regulation of cell growth, development, and metabolism (2). However, a different mechanism of action, termed nongenomic or extranuclear effect, has also been reported, which promotes rapid responses in cells and tissues.

![Western blot analysis of Akt, Rel A, PI3K, GAPDH expression in bone marrow-derived iDCs treated with siRNA for Akt, Rel A, PI3K, or scramble siRNA.](image)

**FIGURE 6. Critical role for Akt and NF-κB in T₃-induced DC maturation and IL-12 production.** A. Western blot analysis of Akt, Rel A, PI3K, TRβ₁, and GAPDH expression in bone marrow-derived iDCs treated with siRNA for Akt1, Rel A, PI3K (p85α), or scramble siRNA. A representative of three independent experiments is shown. B and C, bone marrow-derived iDCs were treated for 18 h with T₃ (5 nM; DC₃), LPS (100 ng/ml; DC_LPS), or left untreated (DC_Control); following siRNA-mediated silencing of Akt, Rel A, PI3K, or introduction of scramble siRNA as described under “Experimental Procedures.” The percentage of IL-12⁺ cells was determined by flow cytometry; histograms are gated on CD11c⁺ cells. Numbers in plots (B and C) indicate the percentage of positively stained cells in gate. Data are from one representative of three independent experiments.
occurs within minutes or even seconds of exposure to TH. Such effects have actually been known for many years, although the underlying mechanisms are still poorly understood (6). Several intracellular pathways have been described to be responsible for TH-mediated cytoplasmic actions, including the MAPK, protein kinase C, and PI3K-protein kinase B/Akt pathways. The co-localization of TRβ1 with Akt, but not with PI3K, as well as the lack of effect of PI3K inhibitors in T3-induced Akt phos-
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phorylation and IL-12 production, supports the concept of a PI3K-independent mechanism of Akt activation. Moreover, the lack of ERK 1/2, p38, or JNK phosphorylation together with the sustained T3-induced Akt phosphorylation even in the presence of a PKA inhibitor disregard the participation of other major signaling pathways in T3 effects. In turn, the co-localization of TRβ1 and Akt was independent of the addition of T3, whereas Akt phosphorylation required T3 binding to its specific receptor. Hence, other molecular and/or biochemical events may be elicited following T3 binding to TRβ1. Several reports suggested PI3K-independent mechanisms of Akt activation induced by various agents, including forskolin, chlorophenylthio-cAMP, prostaglandin-E1, and 8-bromo-cAMP, which were shown to activate Akt through PKA. In this regard, Akt can be activated by a Ca2+ -calmodulin-dependent kinase activity in vitro or by cellular stress through association with Hsp27. In addition, isoproterenol, a β-adrenergic agent, can activate Akt in a wortmannin-resistant manner (33). In turn, many proteins have been reported to interact with Akt to favor relevant biological functions, including Hsp27, in which interaction with Akt leads to inhibition of apoptosis in neutrophils (34). It is unlikely that T3-bound TRβ1 could phosphorylate Akt, as it lacks kinase activity. Nevertheless, the formation of a multiple protein complex associated with T3-bound TRβ1-Akt that includes an enzyme with kinase properties may be responsible for the rapid Akt phosphorylation triggered by T3. However, the precise intracellular events triggered by T3-TRβ1 interactions and/or the molecular identity of this putative Akt kinase remain elusive. Yet, the lack of effect of CHX on T3-induced Akt phosphorylation does not support the involvement of a newly synthesized protein taking part in this process. Recently, other authors also reported T3-mediated cytoplasmic actions in β pancreatic cells through mechanisms involving TRβ1 and Akt activation; yet this effect involved co-localization of PI3K with TRβ1 and was sensitive to PI3K inhibitors (10). On the other hand, although T3 treatment did not induce changes in PPA2-Akt interaction or reduction in PPA2 expression, inhibition of phosphatase activity cannot be completely excluded as an alternative mechanism responsible for T3-induced Akt phosphorylation.

Remarkably, T3-dependent Akt activation was rapidly induced on DCs and lasted for several hours. This finding is of physiological and therapeutic relevance as Akt activation has been shown to be of critical importance for promoting DC survival (35). Hence, manipulation of Akt activation by T3-TRβ1 signaling opens new avenues for therapeutic harnessing of the inherent immunogenicity of DCs to develop more efficient DC-based tumor vaccines (35).

The increased production of IL-12 in T3-treated DCs was completely abolished by pharmacological or siRNA-mediated disruption of either Akt or NF-κB pathways. These results contrast with those obtained using LPS as a stimulus, where only NF-κB inhibitors significantly prevented the increase in the frequency of IL-12-producing CD11c+ DCs (36, 37). In this regard, activation of Akt entails a complex series of events involving additional proteins. When phosphorylated, Akt can rapidly translocate to specific intracellular compartments and amplifies specific signaling processes. This sequential series of events is necessary to generate fully activated Akt and has been demonstrated also in experimental models (10).

In our study, T3 treatment triggered translocation of active Akt to the nucleus, thus allowing this kinase the possibility to further regulate the expression and/or activity of nuclear proteins involved in DC physiology and survival. This finding suggests a dual role of T3-induced Akt activation, acting both at the cytoplasmic and nuclear compartments. Although in the absence of T3, TRβ1 expression was substantially lower in the nucleus compared with the cytoplasm of DCs (17), a significant pool of TRβ1 shuttled to the nucleus following exposure to this thyroid hormone, in agreement with other cell types (29). Hence, the participation of the classical genomic TRβ1 pathway cannot be excluded.

Given a number of reports emphasizing the independence of the TH receptor in T3-mediated cytoplasmic functions (6), a critical issue we wished to address was whether TRβ1 was essential or not for T3-mediated immunostimulatory effects. By means of siRNA-mediated silencing strategies, we found an essential role for TRβ1 in the control of DC maturation, signaling, and function, as shown by reduced expression of cell surface co-stimulatory molecules, diminished IL-12 production, impaired Akt phosphorylation, and unaltered IκB-ε and IκB-α levels following addition of T3 to TRβ1 knockdown iDCs. These findings confirm the relevance of cytoplasmic TRβ1 expression in mediating T3 effects within the DC compartment and predict important deficits in the initiation of adaptive immunity in patients suffering the TH resistance syndrome who carry non-functional TRβ1 (38). In this regard, Baumann et al. (29) demonstrated that TRβ1 rapidly shuttles between the nucleus and...
The cytoplasm. The possible cross-talk between nongenomic and genomic TH signaling is complex and still remains to be fully ascertained; yet these effects appear to act synergistically to modulate cellular processes. The postulated nongenomic signaling pathway may be complemented by nuclear actions of the thyroid hormone, which may amplify the system by generating second messengers and activating multiple signaling cascades. Illustrating this concept, the genomic and nongenomic effects of TH also appear to be strikingly synergistic within the mitochondrial compartment (39). These findings reveal the complexity of the TR signaling and suggest that a cytoplasmic TR with high homology to its nuclear counterpart might be implicated in T₃-induced DC maturation. Small interfering RNAs, designed to specifically act on nuclear TR_{1} mRNA, could silence both cytoplasmic and nuclear TR_{1} without discrimination (data not shown). Furthermore, the commercial monoclonal antibody raised against a nuclear TR (see “Experimental Procedures”) clearly recognized a cytoplasmic protein responsible for T₃ effects, supporting our hypothesis of a cytoplasmic TR with striking similarities to nuclear TR.

The formation of ligand-bound TR complexes is the critical step in the regulation of TH functions and the regulation of TR levels by its specific ligand T₃ is isoform- and cell type-dependent (40). In our study, T₃ treatment induced a significant increase in TR_{1} expression on DCs, and surprisingly, NF-κB inhibitors prevented T₃-induced TR_{1} up-regulation. From the early cloning of the TRB1 gene and the study of its promoter region, several consensus sites have been reported for many transcription factors (31, 41). Here, we identified a functional consensus site for NF-κB located 44 to 52 bp upstream of the transcription start site. Hence, the augmented expression of TR_{1} recorded following exposure of iDCs to T₃ may be achieved, at least in part, through stimulation of NF-κB signaling, thus facilitating a positive regulatory loop in which T₃ regulates TR_{1} expression, and in turn TR_{1} mediates T₃ signaling. On the other hand, it has been demonstrated that classical NF-κB-regulated promoters become derepressed by recruiting chromatin-associated NF-κB kinase, which is responsible for stimulating nuclear export and degradation of the silencing mediator of retinoid and TH receptors and recruitment of both histone acetyltransferase and ATP-dependent remodeling

**FIGURE 9.** T₃ up-regulates TR_{1}, expression on DCs through an NF-κB-dependent mechanism. A, Western blot analysis of TR_{1} expression on DCs treated or not with T₃ (5 nM) for the indicated time periods in the absence or presence of a specific NF-κB inhibitor (BAY 11-7082, left panel) or following siRNA-mediated silencing of Rel A (right panel) as described under “Experimental Procedures.” The expressions of α-tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown as controls of equal loading, and the expression of Rel A is shown as a control of effective silencing. B, ChIP analysis amplifying a 206-bp fragment from the promoter region of the TRB1 gene (pTRB1) encompassing a putative NF-κB consensus site using a p65 immunoprecipitated DNA from DCs treated or not with T₃ (5 nM) for 30 min and 18 h as template DNA (1:100, 1st lane; 1:10, 2nd lane; and 1:1, 3rd lane). Input DNA (1:10,000) is shown (lower blot). C, results from ChIP experiments were analyzed by real time PCR as described under “Experimental Procedures” and expressed as fold change in occupancy (relative units) on DCs treated or not with T₃ (5 nM) for 30 min or 18 h. Data are the means ± S.D. of three independent experiments.* p < 0.01 versus DC₃.
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complexes (42). This mechanism may also contribute to the increased TRβ1 expression on DCs after T₃-induced NF-κB activation. Interestingly, other nuclear receptors also appear to be regulated by NF-κB, as this transcription factor specifically binds the −574/−565 promoter region of the androgen receptor, which mediates repression of its transcription (43).

In conclusion, this study highlights a novel mechanism by which T₃/TRβ1 signaling modulates DC maturation through activation of an Akt- and NF-κB-dependent but PI3K-independent pathway. Finally, our data underscore a possible regulatory loop by which T₃ signaling promotes further expression of TRβ1, through mechanisms involving functional interactions of the NF-κB transcription factor to the promoter region of the TRB1 gene.

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