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Nuclear Factor (NF)-κB-dependent Thyroid Hormone Receptor β_1 Expression Controls Dendritic Cell Function via Akt Signaling*[S]

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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) β_1 signaling in modulating dendritic cell (DC) physiology and the intracellular mechanisms underlying these immunoregulatory effects. Exposure of DCs to triiodothyronine (T_3) resulted in a rapid and sustained increase in Akt phosphorylation independently of phosphatidylinositol 3-kinase activation, which was essential for supporting T_3-induced DC maturation and interleukin (IL)-12 production. This effect was dependent on intact TRβ_1 signaling as small interfering RNA-mediated silencing of TRβ_1 expression prevented T_3-induced DC maturation and IL-12 secretion as well as Akt activation and IκB-ε degradation. In turn, T_3 up-regulated TRβ_1 expression through mechanisms involving NF-κB, suggesting an autocrine regulatory loop to control hormone-dependent TRβ_1 signaling. These findings were confirmed by chromatin immunoprecipitation analysis, which disclosed a new functional NF-κB consensus site in the promoter region of the *TRB1* gene. Thus, a T_3-induced NF-κB-dependent mechanism controls TRβ_1 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 crossroads of the immune-endocrine circuits.

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 TRα_1, TRα_2, TRβ_1, and TRβ_3 isoforms are widely expressed, whereas TRβ_2 is predominantly restricted to the hypothalamus-pituitary axis (2). Recent emerging evidence has also characterized the interactions of TR with co-repressor 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 (T_3) and several co-activator proteins that exhibit histone acetylase 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 TH-dependent effects mediated by the cell surface $\alpha_v\beta_3$ integrin (6). Several cytoplasmic T_3 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 TRβ_1 and activation of PI3K-p85 (10).

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

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[5] The abbreviations used are: TH, thyroid hormone; TR, thyroid hormone receptor; DC, dendritic cell; T_3, triiodothyronine; PI3K, phosphatidylinositol 3-kinase; IL, interleukin; siRNA, small interfering RNA; iDC, immature DC; LPS, lipopolysaccharide; 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.

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* or *in vivo* 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 stimulatory capacity and IL-12 production, which result in the induction of protective Th1 immunity (15). Recently, we have provided the first evidence indicating a role for TH in the control of DC maturation (17). Our initial results demonstrated the expression of TR, mainly the β_1 isoform, on bone marrow-derived murine DCs and the role of T_3 in driving DC maturation (17).

In this study, we investigated the signaling pathways leading to T_3 effects within the DC compartment, their impact in the immunogenicity of these cells, and the relevance of TRβ_1 signaling in this process. Our results demonstrate a novel link among NF-κB-dependent TRβ_1 expression, T_3-induced Akt 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 treatment with resin AG-1-X8 (Bio-Rad) in the presence of granulocyte-macrophage colony-stimulating factor from supernatants of the J558 cell line, and fed every 2 days. At day 10 of cell culture, >85% of the harvested cells expressed MHC II, CD40, CD80, and CD11c but not Gr-1. Immature DCs (iDCs) were cultured with T_3 (5 nM, DC_{T3}) or LPS (100 ng/ml; *Escherichia coli* strain 0111:B4; Sigma; DC_{LPS}). Parallel cultures were maintained without stimuli and used as controls ($DC_{Control}$). T_3 was purchased from Sigma and prepared according to the manufacturer's recommended protocol. The concentration of T_3 used in the experiments led to 60–80% of thyroid receptor occupancy, a situation in which most of the biological actions of TH take place under physiological conditions (2). To rule out endotoxin contamination of the T_3 preparation, we checked endotoxin content after reconstitution of the hormone, which raised levels lower than 0.03 IU/ml (limit of detection) by using the *Limulus* amebocyte lysate assay (Sigma).

Immunoblot Analysis—SDS-PAGE and blot analysis were performed essentially as described (19). Anti-TRβ_1 (mouse monoclonal antibody), anti-Akt 1/2/3, anti-PI3K p85α (rabbit polyclonal antibodies), anti-NF-κB-p65 (anti-Rel A; mouse monoclonal antibody), and anti-HDAC1 (mouse monoclonal antibody) were obtained from Santa Cruz Biotechnology. Anti-TRβ_1 (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-ϵ and 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 (1.5×10^6 cells/300 μl) in the presence of T_3 (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-p3, 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-TRβ_1 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 Flowview 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 phenylmethylsulfonyl 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-TRβ_1 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-TRβ_1 (1:500) mouse antibody, respectively. The membranes were stripped and exposed to a rabbit anti-TRβ_1 (C4) (1:500) or a mouse anti-TRβ_1 (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 TRβ_1 expression, DCs were fixed with 1% (w/v) paraformaldehyde, treated with fluorescence-activated cell sorter permeabilizing solution (BD Biosciences), stained with a mouse anti-TRβ_1 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 T_3 or LPS in the absence or presence of 1 μM BAY 11-7082 or 2 nM 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 FACSAria 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 multiwell 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 (2×10^6 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 M 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,

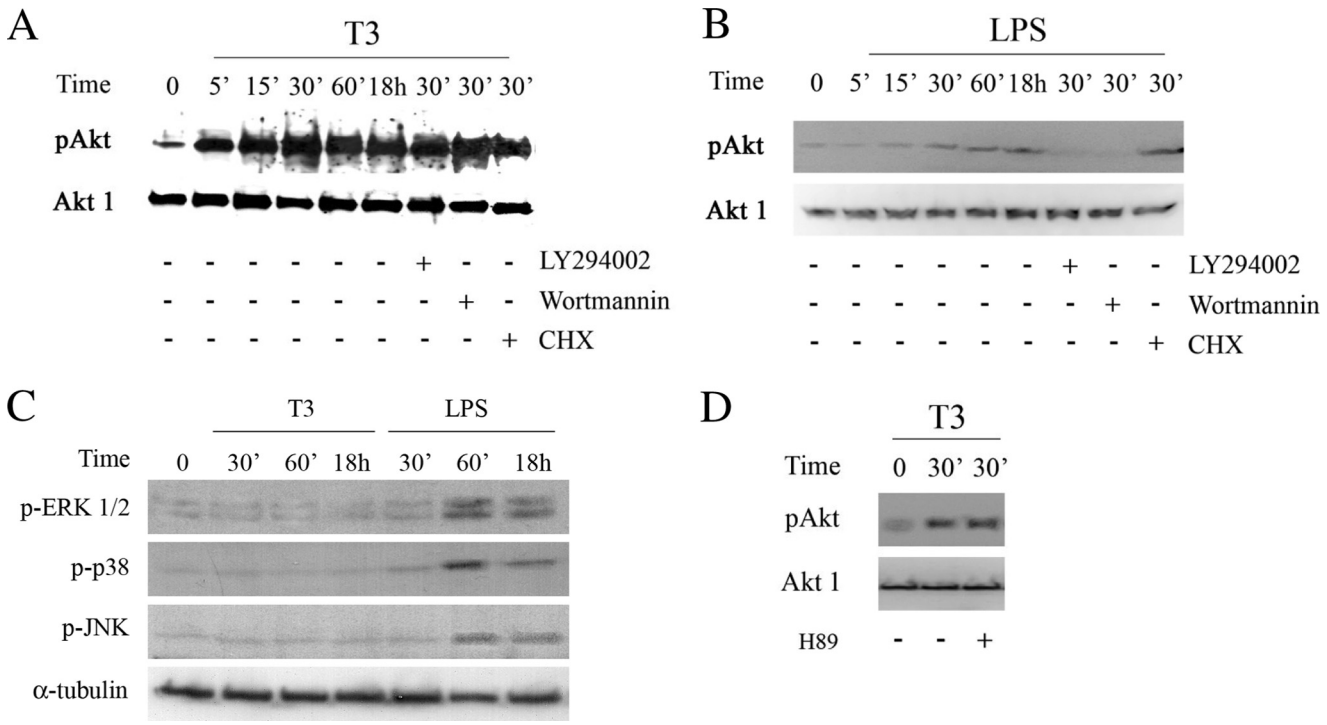


FIGURE 1. **T_3 induces activation of Akt in a PI3K-independent manner.** Bone marrow-derived iDCs differentiated with granulocyte-macrophage colony-stimulating factor for 7 days were exposed to T_3 (5 nM) or LPS (100 ng/ml). After different time periods, cells were harvested, and lysates were prepared and analyzed by Western blot for phosphorylated (*pAkt*) or unphosphorylated Akt (*Akt 1*), phosphorylated ERK 1/2, p38, or JNK. In parallel DCs were preincubated with LY249002 or wortmannin (PI3K inhibitors), CHX, or H89 (PKA inhibitor) for 30 min before exposure to T_3 or LPS. *A,* T_3 induces rapid phosphorylation of Akt (after 5 min of exposure), which persists for at least 18 h in a PI3K- and CHX-insensitive manner. *Lower panel* shows the same blot probed for total Akt to check equal loading of samples. *B,* phosphorylation of Akt occurs within 15 min following exposure to LPS and is inhibited by preincubation with specific PI3K inhibitors. *C,* in contrast to LPS, T_3 is unable to induce phosphorylation of ERK 1/2, p38, or JNK on DCs. *Lower panel* shows the same blot probed for α-tubulin to check equal loading of samples. *D,* T_3 induces Akt phosphorylation (after 30 min of exposure) in a PKA-insensitive manner as shown by incorporation of H89 to DC cultures. *Lower panel* shows the same blot probed for total Akt to check equal loading of samples. *Blots* are representative of six independent experiments with similar results.

1:10, and 1:1 of the immunoprecipitated material and 1:10,000 of the input material. PCR products were resolved in 2% (w/v) agarose gel and visualized with 0.004 μg/ml ethidium bromide. Oligonucleotide PCR primers used for ChIP analysis amplified a DNA fragment (206 kb) homologous to the promoter of the *TRB1* gene encompassing the putative NF-κB consensus site (sense, TGATCAGAGTGAGGCTGTGC, and antisense, GCTGGTGGAGTCCTGAGTTC).

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 ΔC_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 $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value for the T_3-treated sample from the ΔC_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 $-\Delta\Delta C_T$ 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

T_3 Triggers PI3K-independent Akt Activation in Bone Marrow-derived Mouse DCs—Given the immunostimulatory effects of T_3 and the preferential cytoplasmic localization of TRβ_1

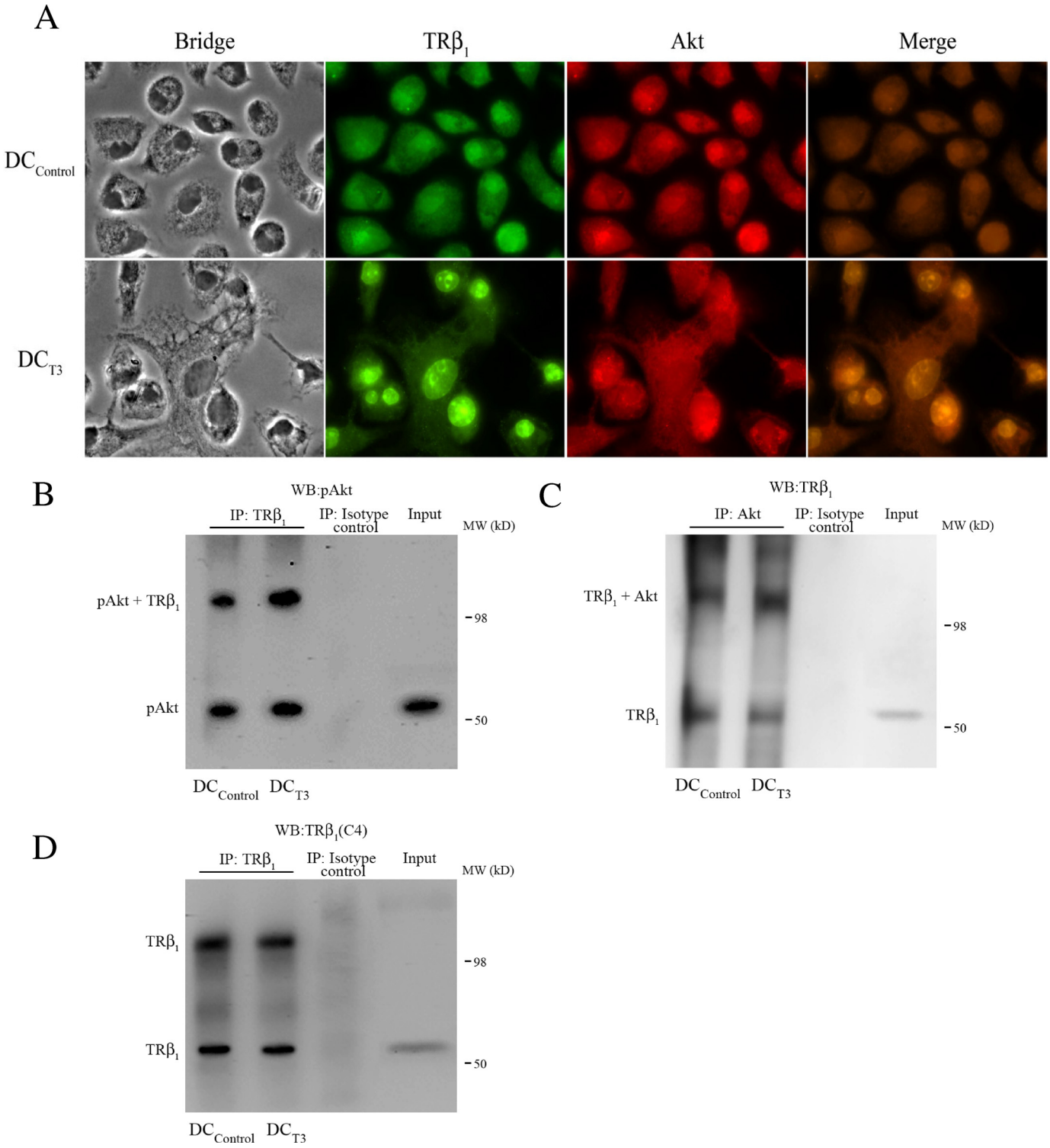


FIGURE 2. **TRβ_1 co-localizes with Akt in the cytoplasm of iDCs in a ligand-independent manner.** Bone marrow-derived iDCs were cultured for 18 h with or without T_3 (5 nM) (DC_{T3} and $DC_{Control}$, respectively). *A,* immunofluorescence analysis. DCs were immunostained for TRβ_1 (*green fluorescence*) and Akt (*red fluorescence*). All images were acquired and analyzed by confocal microscopy as described under "Experimental Procedures." *B–D,* co-immunoprecipitation analysis. Immunoprecipitation (*IP*) was performed in whole cell lysates using anti-TRβ_1 (*B* and *D*), anti-Akt (*C*), or an irrelevant isotype control antibody. Western blot (*WB*) analyses for pAkt (*B*), TRβ_1 (*C*), and TRβ_1 (C4) (*D*) were performed as described under "Experimental Procedures." The identities of the bands were corroborated with the input control. *Blots* are representative of three independent experiments.

on DCs (17), in this study we investigated the intracellular pathways triggered by TRβ_1 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 T_3-stimulated (DC_{T3})and control ($DC_{Control}$) DCs. Bone marrow-derived iDCs were cultured in the presence or in the absence of T_3 (5 nM) for 5, 15, 30, and 60 min and 18 h. A significant increase in

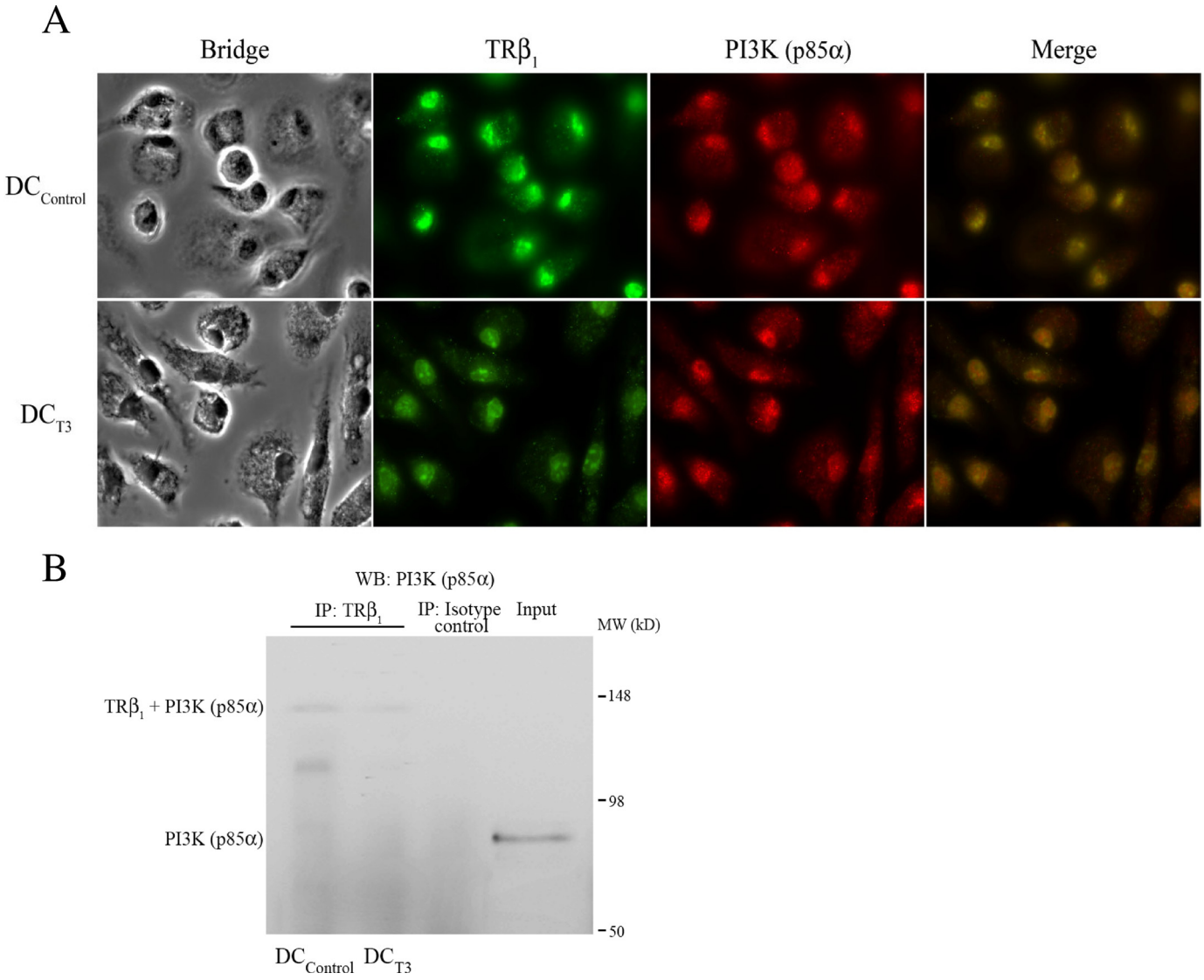


FIGURE 3. **TRβ_1 does not co-localize with PI3K-p85 in bone marrow-derived iDC.** Bone marrow-derived iDCs were cultured for 18 h with or without T_3 (5 nM) (DC_{T3} and $DC_{Control}$, respectively). *A,* immunofluorescence staining. DCs were immunostained for TRβ_1 (*green fluorescence*) and PI3K-p85 (*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-TRβ_1 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.

Ser-473 phosphorylation of Akt was detected as early as 5 min following exposure of DCs to T_3 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. 1*A*). Remarkably, T_3-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. 1*A*). In turn, T_3 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. 1*A*). Of note, Akt phosphorylation was barely observed on DCs exposed to LPS (Fig. 1*B*).

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 T_3 as well as the effect of the inhibition of cAMP-activated protein kinase (protein kinase A (PKA)) signaling on T_3-induced Akt phosphorylation. In contrast to LPS, T_3 could not trigger ERK 1/2, p38, or JNK phosphorylation when added for different time periods to DC cultures (Fig. 1*C*). Moreover, H89, a potent inhibitor of PKA, could not prevent T_3-induced Akt phosphorylation (Fig. 1*D*). 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 T_3-mediated up-regulation of Akt. Of note, the levels of PP2A C were not reduced following treatment of DCs with T_3. On the contrary, PP2A C was slightly increased compared with DCs cultured with medium alone (supplemental Fig. 1). Moreover, exposure to T_3 did not alter the normal interaction between Akt and PP2A C (supplemental Fig. 2), disregarding, at least in part, the participation of this serine/threonine phosphatase in T_3-mediated induction of Akt. Collectively, these results support the concept of an Akt-dependent PI3K-independent mechanism underlying T_3 modulation of DC physiology. In

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 T_3 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 T_3 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 T_3, supporting the notion of nongenomics actions of T_3 involving Akt activation. Consistent with the lack of effect of PI3K inhibitors on T_3-induced Akt phosphorylation, we could observe no co-localization (Fig. 3*A*) or physical interaction (Fig. 3*B*) between TRβ1 and PI3K, neither in the absence nor in the presence of T_3, 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 T_3 to trigger rapid recruitment of pAkt (Ser-473) to the nucleus following exposure to T_3 (Fig. 4). Moreover, a significant proportion of TRβ1 shuttled to the nuclear fraction (Fig. 2*A* 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 T_3 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 T_3-mediated effects was substantiated by functional experiments. These involved evaluation of cell surface phenotypic markers and IL-12 production on iDCs exposed to T_3 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 T_3 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. 5*A*). Remarkably, this effect was prevented when DCs were matured with either T_3 or LPS in the presence of a specific NF-κB inhibitor (BAY 11-7082). However, pharmacological disruption of Akt specifically prevented T_3 but not LPS effects (Fig. 5*A*), suggesting that T_3 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 T_3 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

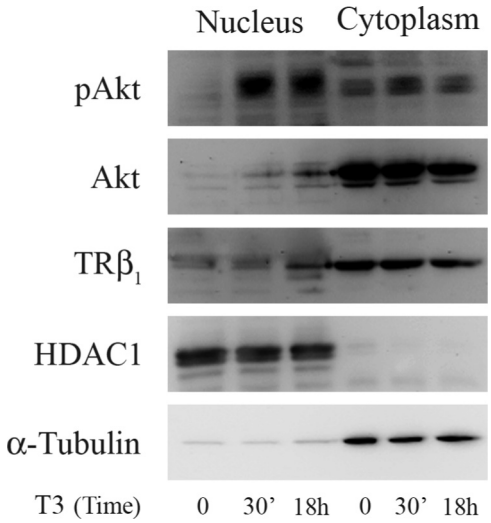


FIGURE 4. **T_3 induces nuclear translocation of activated Akt.** Western blot analysis of phosphorylated Akt (*pAkt*), Akt, and TRβ1 in protein extracts obtained from nuclear and cytoplasmic fractions of DCs exposed to T_3 for 30 min or 18 h. The expression of HDAC1 (nuclear) and α-tubulin (cytosolic) was analyzed as controls to check equal gel loading and exclude contamination of the nuclear fraction with cytoplasmic components. However, minimal cytoplasmic contamination of the nuclear fraction, as shown by α-tubulin expression, did not alter the conclusions raised. *Blots* are representative of three independent experiments.

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 T_3-induced DC maturation, as shown by cell surface phenotypic markers (Fig. 6*B*) and IL-12 production (Fig. 6*C*). In contrast, T_3 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 T_3 effects (Fig. 6, *B* and *C*). The specificity and efficacy of all siRNAs used are shown in Fig. 6*A*. Thus, T_3 delivers maturation signals to iDCs via selective modulation of Akt (independently of PI3K) and NF-κB.

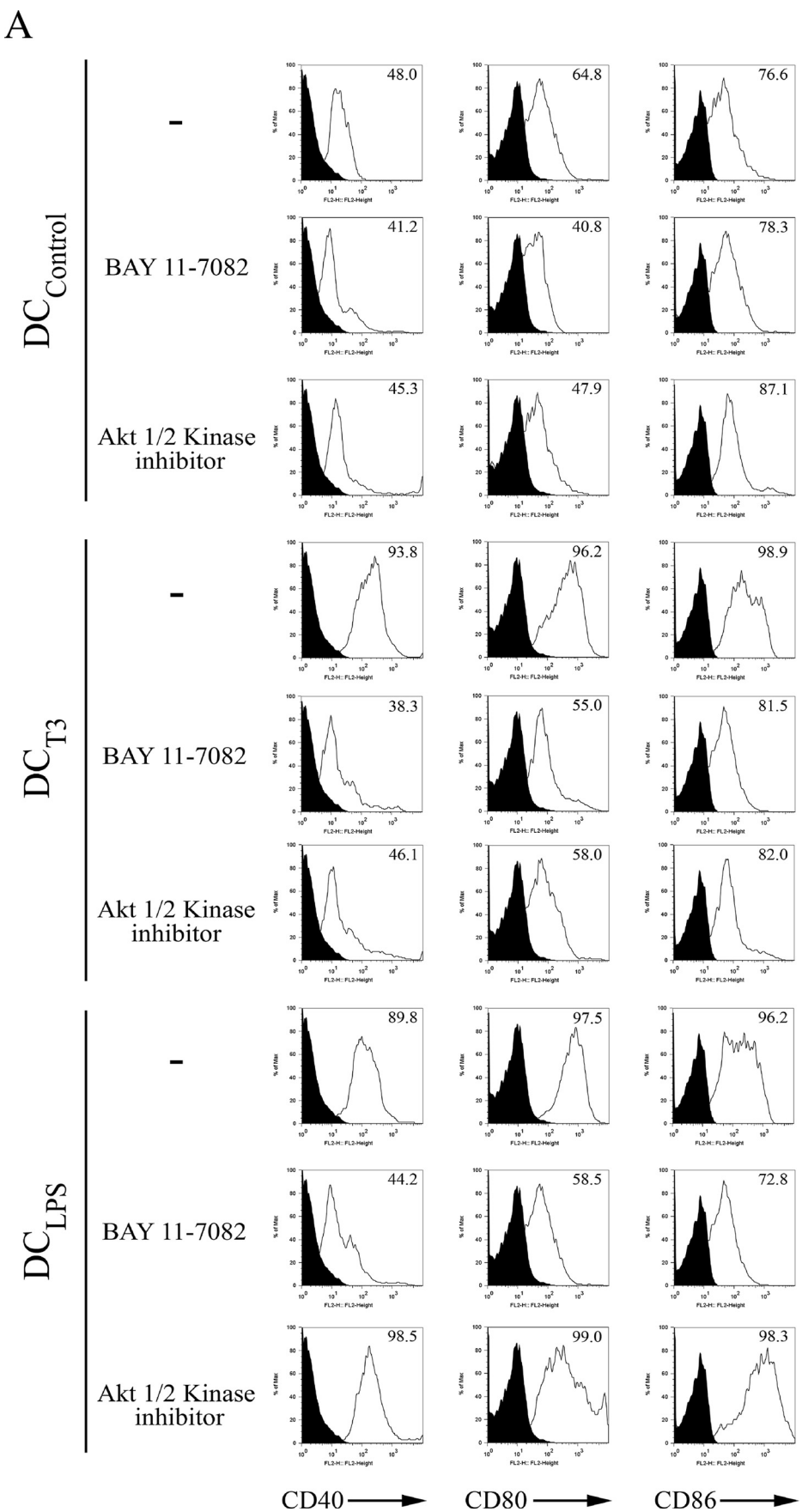
Critical Role of TRβ1 Signaling in T_3-induced DC Maturation—To investigate whether TRβ1 is critical in T_3-induced DC maturation, we interrupted TRβ1 signaling on these cells by siRNA-mediated silencing strategies. Transfection of TRβ1-siRNA but not scramble siRNA almost completely abrogated TRβ1 expression, as shown by flow cytometric analysis of permeabilized DCs (Fig. 7*A*) and immunoblot analysis of total cell lysates (Fig. 7*B*). More importantly, siRNA-mediated TRβ1 silencing substantially prevented T_3-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. 7*C*). Furthermore, blockade of TRβ1 signaling completely prevented T_3-induced IL-12 secretion (Fig. 7*D*), 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 T_3-induced Akt and NF-κB activation, we stimulated control or

TRβ$_1$-siRNA-transfected DCs with T_3 for 30 min or 18 h and analyzed Akt phosphorylation and IκB-ϵ degradation. Remarkably, TRβ$_1$ silencing completely abrogated T_3-induced Akt phosphorylation and IκB-ϵ degradation, although it did not alter basal Akt or IκB-ϵ expression. (Fig. 8). Likewise, TRβ$_1$ or Akt silencing prevented T_3-induced IκB-α degradation (supplemental Fig. 4). Taken together, these results imply a critical role of intact TRβ$_1$ expression in coupling T_3 binding, Akt- and NF-κB-dependent signaling, and DC maturation.

T_3 Regulates TRβ$_1$ Expression on DCs via NF-κB—It is well established that TRβ$_1$ expression is differentially regulated by T_3 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 T_3 on TRβ$_1$ expression on iDCs. Notably, treatment of iDCs with T_3 (5 nM) for 30 min considerably increased TRβ$_1$ protein expression, which remained elevated up to 18 h and returned to control levels after 24 h of exposure to T_3 (Fig. 9*A*) (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 T_3-induced up-regulation of TRβ$_1$ (Fig. 9*A*, *left* and *right panels*), suggesting involvement of this transcription factor as a regulator of T_3-induced TRβ$_1$ 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



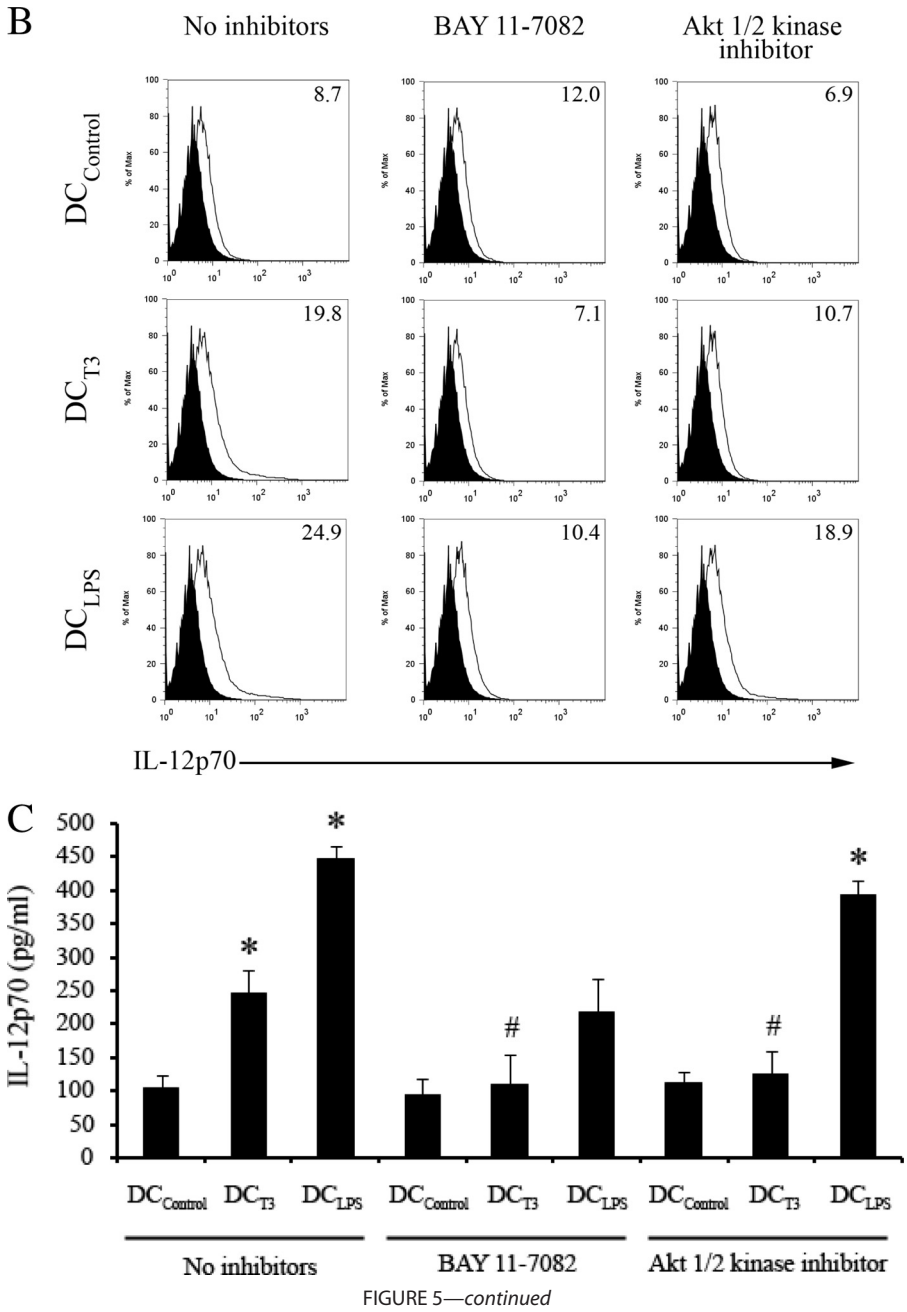


FIGURE 5—*continued*

obtained from anti-NF-κB (p65) immunoprecipitated chromatin revealed a positive signal (Fig. 9*B*). Remarkably, this signal was considerably amplified following treatment of iDCs with T_3 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 T_3-induced NF-κB-dependent regulation of TRβ_1 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 T_3 in promoting DC maturation and Th1-type cytokine secretion (17). As the T_3-TRβ_1 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 TRβ_1 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. **T_3 promotes DC maturation and IL-12 production via Akt- and NF-κB-dependent pathways.** Bone marrow-derived iDCs were treated with T_3 (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 T_3 (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 T_3 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-12^+ 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.

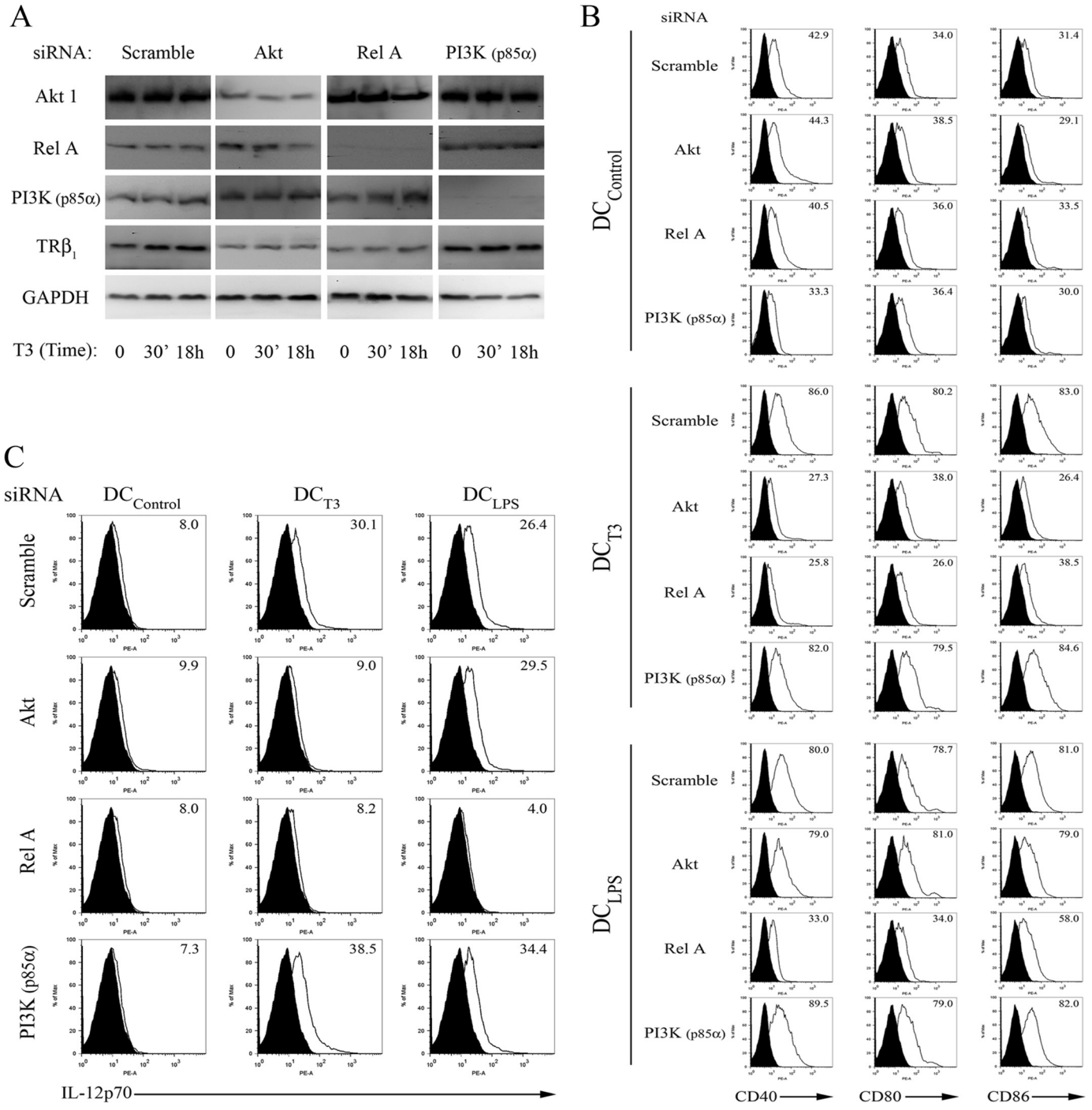


FIGURE 6. **Critical role for Akt and NF-κB in T_3-induced DC maturation and IL-12 production.** *A,* Western blot analysis of Akt 1, Rel A, PI3K, TRβ_1, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression in bone marrow-derived iDCs treated with siRNA for Akt1, Rel A, PI3K (p85α), or scramble siRNA. A representative of three independent experiments is shown. *B* and *C,* bone marrow-derived iDCs were treated for 18 h with T_3 (5 nM; DC_{T3}), 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." At the end of the experimental period, DCs were washed and processed for flow cytometry of cell surface markers (*B*) and intracytoplasmic IL-12 staining (*C*). *B,* flow cytometry analysis of phenotypic markers (CD40, CD80, and CD86) of DCs treated or not with T_3 (5 nM) or LPS (100 ng/ml) (*black histograms*, nonspecific binding determined with isotype-matched control antibodies; *white histograms*, phenotypic markers). *C,* intracytoplasmic detection of IL-12p70. Following incubation of DCs with T_3 or LPS, cells were incubated with brefeldin A for 4 h, fixed, permeabilized, and stained with anti-IL-12p70 antibody 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.

The ability of T_3 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

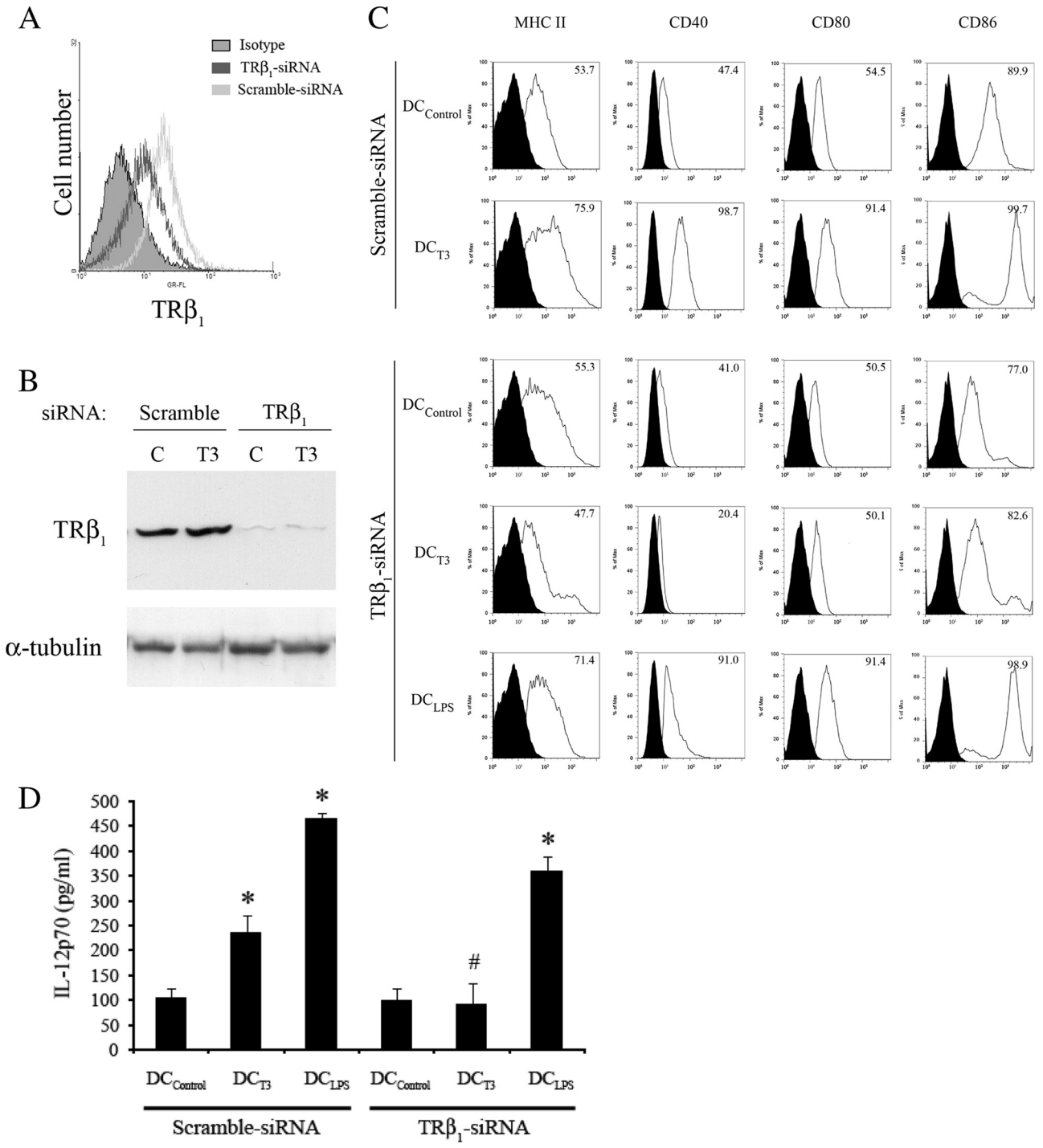


FIGURE 7. **Critical role of TRβ_1 in T_3-induced DC maturation and IL-12 production.** *A,* flow cytometric analysis of TRβ_1 expression on bone marrow-derived iDCs following siRNA-mediated TRβ_1 signaling (TRβ_1-siRNA) or transfection with scramble siRNA as described under "Experimental Procedures." Binding of an irrelevant isotype control antibody is also shown. *B,* Western blot analysis of TRβ_1 expression on iDCs transfected with scramble siRNA or TRβ_1-siRNA and then treated with or without T_3 (5 nM) for 18 h. Expression of α-tubulin was assessed as a control of equal loading. *Blots* are representative of three independent experiments with similar results. *C,* flow cytometric analysis of cell surface phenotypic markers (MHC II, CD40, CD80, and CD86) on DCs treated (DC_{T3} or DC_{LPS}) or not ($DC_{Control}$) with T_3 (5 nM) or LPS (100 ng/ml) for 18 h following siRNA-mediated TRβ_1 silencing (*black histograms*, nonspecific binding determined with isotype-matched control antibodies; *white histograms*, phenotypic markers); histograms are gated on $CD11c^+$ cells. *Numbers* in plot indicate the percentage of positively stained cells in gate. Representative histograms of six independent experiments are shown. *D,* enzyme-linked immunosorbent assay of IL-12p70 production in supernatants of DCs treated (DC_{T3} or DC_{LPS}) or not ($DC_{Control}$) with T_3 (5 nM) or LPS (100 ng/ml), respectively, for 18 h following siRNA-mediated TRβ_1 silencing. *, $p < 0.05$ *versus* scramble-siRNA $DC_{Control}$; #, $p < 0.05$ *versus* scramble-siRNA DC_{T3} (Student's *t* test). Data are the means ± S.D. of three independent experiments. *C,* control.

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 TRβ_1 with Akt, but not with PI3K, as well as the lack of effect of PI3K inhibitors in T_3-induced Akt phos-

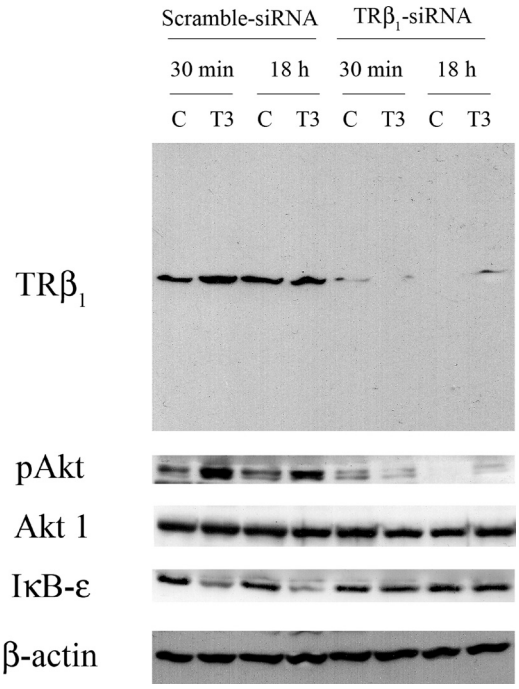


FIGURE 8. **Critical role of TRβ_1 in T_3-induced Akt and NF-κB activation.** Western blot analysis of phosphorylated Akt and IκB-ε on iDCs treated or not with T_3 (5 nM) for 30 min or 18 h following siRNA-mediated silencing of TRβ_1 (TRβ_1-siRNA) or introduction of scramble siRNA. Unphosphorylated Akt and β-actin are shown to check equal loading of samples. *Blots* are representative of three independent experiments. *C*, control.

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 T_3-induced Akt phosphorylation even in the presence of a PKA inhibitor disregard the participation of other major signaling pathways in T_3 effects. In turn, the co-localization of TRβ_1 and Akt was independent of the addition of T_3, whereas Akt phosphorylation required T_3 binding to its specific receptor. Hence, other molecular and/or biochemical events may be elicited following T_3 binding to TRβ_1. Several reports suggested PI3K-independent mechanisms of Akt activation induced by various agents, including forskolin, chlorophenylthio-cAMP, prostaglandin-E_1, and 8-bromo-cAMP, which were shown to activate Akt through PKA. In this regard, Akt can be activated by a Ca^{2+}-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 T_3-bound TRβ_1 could phosphorylate Akt, as it lacks kinase activity. Nevertheless, the formation of a multiple protein complex associated with T_3-bound TRβ_1-Akt that includes an enzyme with kinase properties may be responsible for the rapid Akt phosphorylation triggered by T_3. However, the precise intracellular events triggered by T_3-TRβ_1 interactions and/or the molecular identity of this putative Akt kinase remain elusive. Yet, the lack of effect of CHX on T_3-induced Akt phosphorylation does not support the involvement of a newly synthesized protein taking part in this process. Recently, other authors also reported T_3-mediated cytoplasmic actions in β pancreatic cells through mechanisms involving TRβ_1 and Akt activation; yet this effect involved co-localization of PI3K with TRβ_1 and was sensitive to PI3K inhibitors (10). On the other hand, although T_3 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 T_3-induced Akt phosphorylation.

Remarkably, T_3-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 T_3-TRβ_1 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 T_3-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, T_3 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 T_3-induced Akt activation, acting both at the cytoplasmic and nuclear compartments. Although in the absence of T_3, TRβ_1 expression was substantially lower in the nucleus compared with the cytoplasm of DCs (17), a significant pool of TRβ_1 shuttled to the nucleus following exposure to this thyroid hormone, in agreement with other cell types (29). Hence, the participation of the classical genomic TRβ_1 pathway cannot be excluded.

Given a number of reports emphasizing the independence of the TH receptor in T_3-mediated cytoplasmic functions (6), a critical issue we wished to address was whether TRβ_1 was essential or not for T_3-mediated immunostimulatory effects. By means of siRNA-mediated silencing strategies, we found an essential role for TRβ_1 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 T_3 to TRβ_1 knockdown iDCs. These findings confirm the relevance of cytoplasmic TRβ_1 expression in mediating T_3 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 TRβ_1 (38). In this regard, Baumann *et al.* (29) demonstrated that TRβ_1 rapidly shuttles between the nucleus and

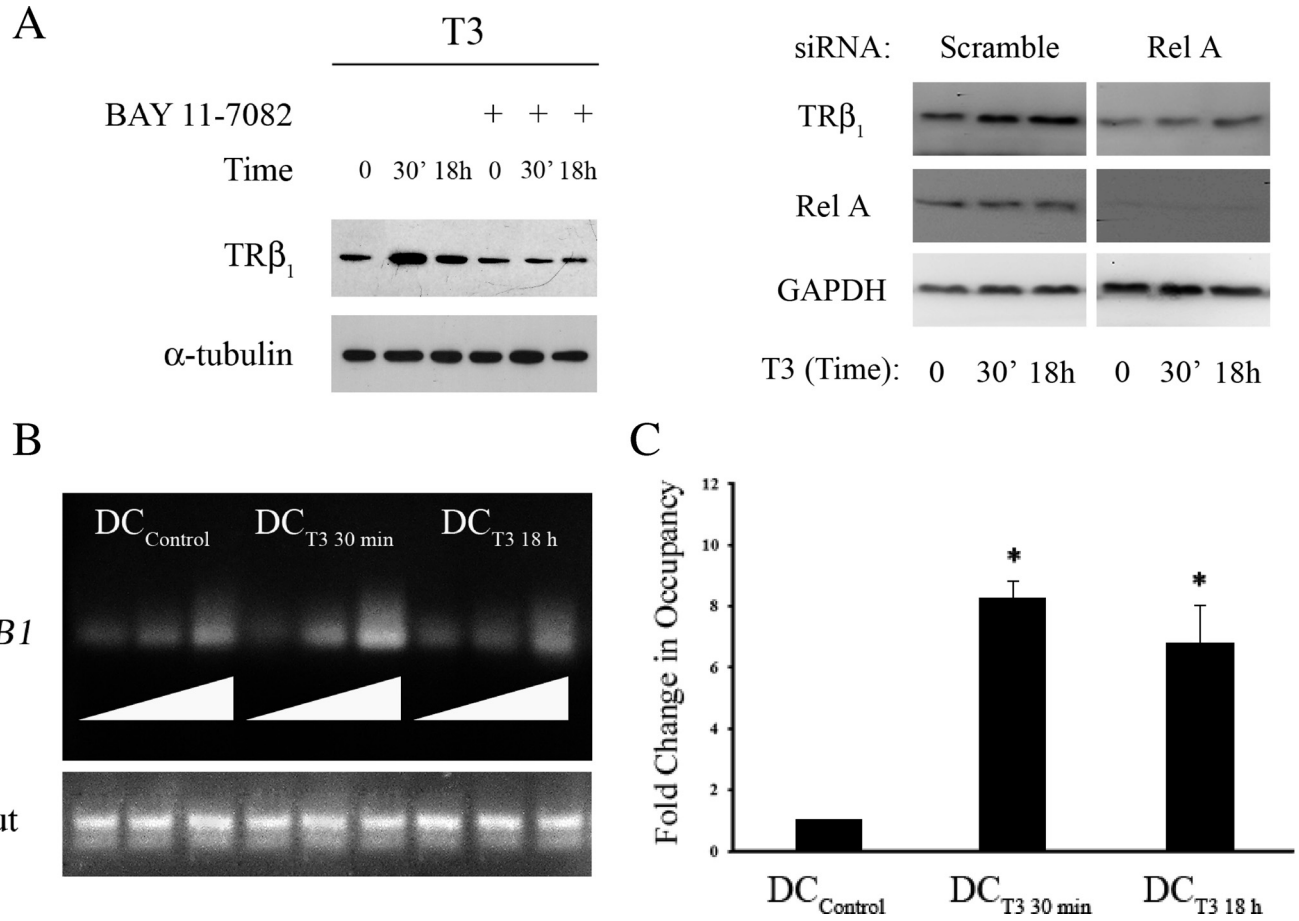


FIGURE 9. **T_3 up-regulates TRβ_1 expression on DCs through an NF-κB-dependent mechanism.** *A,* Western blot analysis of TRβ_1 expression on DCs treated or not with T_3 (5 nM) for the indicated time periods in the absence or presence of a specific NF-κB inhibitor (BAY 11-7082, *left panel*) or following 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. *Blots* are representative of four independent experiments with similar results. *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 T_3 (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 T_3 (5 nM) for 30 min or 18 h. Data are the means ± S.D. of three independent experiments. *, $p < 0.01$ *versus* $DC_{Control}$ (Student's *t* test).

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 T_3-induced DC maturation. Small interfering RNAs, designed to specifically act on nuclear TRβ_1 mRNA, could silence both cytoplasmic and nuclear TRβ_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 T_3 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 T_3 is isoform- and cell type-dependent (40). In our study, T_3 treatment induced a significant increase in TRβ_1 expression on DCs, and surprisingly, NF-κB inhibitors prevented T_3-induced TRβ_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 −644 to −652 bp up to the starting ATG transcription site. Hence, the augmented expression of TRβ_1 recorded following exposure of iDCs to T_3 may be achieved, at least in part, through stimulation of NF-κB signaling, thus facilitating a positive regulatory loop in which T_3 regulates TRβ_1 expression, and in turn TRβ_1 mediates T_3 signaling. On the other hand, it has been demonstrated that classical NF-κB-regulated promoters become derepressed by recruiting chromatin-associated IκB kinase α, 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

complexes (42). This mechanism may also contribute to the increased TRβ_1 expression on DCs after T_3-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 T_3-TRβ_1 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 T_3 signaling promotes further expression of TRβ_1 through mechanisms involving functional interactions of the NF-κB transcription factor to the promoter region of the *TRB1* gene.

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