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Antitumor Responses Stimulated by Dendritic Cells Are Improved by Triiodothyronine Binding to the Thyroid Hormone Receptor β

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Abstract

Bidirectional cross-talk between the neuroendocrine and immune systems orchestrates immune responses in both physiologic and pathologic settings. In this study, we provide *in vivo* evidence of a critical role for the thyroid hormone triiodothyronine (T3) in controlling the maturation and antitumor functions of dendritic cells (DC). We used a thyroid hormone receptor (TR) β mutant mouse (TRβPV) to establish the relevance of the T3-TRβ system *in vivo*. In this model, TRβ signaling endowed DCs with the ability to stimulate antigen-specific cytotoxic T-cell responses during tumor development. T3 binding to TRβ increased DC viability and augmented DC

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No potential conflicts of interest were disclosed.

migration to lymph nodes. Moreover, T3 stimulated the ability of DCs to cross-present antigens and to stimulate cytotoxic T-cell responses. In a B16-OVA mouse model of melanoma, vaccination with T3-stimulated DCs inhibited tumor growth and prolonged host survival, in part by promoting the generation of IFNγ-producing $CD8^+$ T cells. Overall, our results establish an adjuvant effect of T3-TRβ signaling in DCs, suggesting an immediately translatable method to empower DC vaccination approaches for cancer immunotherapy

Introduction

Reciprocal regulation of neuroendocrine and immune systems preserves homeostasis and orchestrates coordinated responses in physiologic and pathologic settings. The attribute of sharing common ligands (hormones and cytokines) and receptors between these two systems allows bidirectional communication and offers additional opportunities for therapeutic intervention (1).

Thyroid hormones are critical regulators of cellular differentiation, growth, and metabolism in virtually all tissues. Cellular activity of thyroid hormone is usually classified as genomic (nuclear) and nongenomic (initiated either in the cytoplasm or at the plasma membrane). The genomic mechanism of thyroid hormone action requires the participation of active thyroid hormone: triiodothyronine (T3) and its nuclear receptors (TR). TRs are codified by *TRA* and *TRB* genes that are expressed in four major isoforms: $TR\alpha_1$, $TR\beta_1$, $TR\beta_2$, and $TR\beta_3$, whereas other non-T3 binding isoforms are also expressed. In addition, nongenomic actions of thyroid hormone have also been recognized (2).

Several transgenic mouse models expressing TR mutants have been developed to understand whether TR isoforms can mediate specific functions *in vivo* (3–5). In particular, TRβ mutant knock-in mice harboring a frame-shift mutation in the last 14 carboxyl-terminal amino acids of $TR\beta_1$ (TRβPV) have been generated (3). The TRβPV mutation was initially identified in a patient bearing the syndrome of thyroid hormone resistance. *In vitro* characterization of PV mutant showed the complete loss of T3-binding and transactivation activities, interfering also with wild-type TR activity (6). TRβPV, as well as other mouse models, has enabled the demonstration that TRs could function as tumor suppressors, revealing how loss of normal functions of TRs either by deletion or by mutation contributes to cancer development, progression, and metastasis (7).

Dendritic cells (DC) are specialized innate immune cells endowed with a unique capacity to orchestrate adaptive immunity. These cells are the main antigen-presenting cells (APC) that recognize, process, and present antigens to naïve T cells for the induction of antigen-specific immune responses. The recognition of lipopolysaccharide (LPS) or other non-self antigens promotes DC maturation through pattern recognition receptors. Matured DCs alter the expression of chemokine receptors and migrate to T-cell zones in secondary lymphoid organs where they present antigens to Naïve T cells (8). Naïve $CD8^+$ T cells are then instructed to proliferate and mount cytotoxic T-cell responses. Interestingly, APC can process exogenous antigens and present them in the context of MHC-I molecules, a process termed “cross-presentation” that is crucial for the induction of protective immunity against viruses and tumors (9).

DC-based cancer immunotherapeutic strategies have been widely used to engender $CD8^+$ T-cell responses using patients' own DCs loaded with tumor-associated antigens *ex vivo* (10). However, the success is frequently limited as activated DCs have a short half-life in lymph nodes, and antigen processing and presentation from dead DCs may induce T-cell tolerance. Therefore, increasing DC survival and immunogenicity represents a major challenge in vaccination strategies (11). Interestingly, increased phosphorylation of Akt has been shown to enhance DC survival and potentiate DC-based immunotherapy (12).

The direct effects of thyroid hormone on T cells, via either genomic or nongenomic mechanisms, have been studied by many authors (13, 14) in both primary T lymphocytes and lymphoma T-cell lines. However, studies on the impact of thyroid hormone in the initiation of adaptive immune responses are just emerging. We showed that expression of TRs, mainly the β1 isoform, contributes to DC maturation and Th1-type cytokine secretion induced by physiologic levels of T3 (15). Mechanistically, this effect involved activation of Akt and NF-κB pathways (16) and was counteracted by glucocorticoids (17).

In this study, we investigated in TRβPV mice the involvement of TRβ in T3-induced modulation of DC function. Particularly, we examined the impact of T3-TRβ signaling in *in vivo* cytotoxicity and *in vitro* antigen cross-presentation. Finally, we studied the effects of T3 in antigen-specific DC-based immunotherapy in the B16 melanoma model.

Materials and Methods

Mice

Wild-type (WT) female C57BL/6 mice (B6; H-2b) were obtained from Ezeiza Atomic Center (Argentina). Homozygous TRβPV C57BL/6 mutant mice (TRβPV) were obtained and geno-typed as described (6). Mice were maintained under specific pathogen-free conditions and used at 6 to 10 weeks old. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH and the local institutional animal care committee.

Cell preparation and culture

Immature bone marrow dendritic cells (iDC) were obtained from C57BL/6 WT or TRβPV mice as described (15). For primary DCs, spleens and lymph nodes were incubated with collagenase D and then mashed through a cell strainer. DCs were sorted as described (18) into $F4/80^-$ $CD11b^+$ $CD11c^+$ $B220^-$ $MHC\text{-}II^+$ $LY6C^-$ conventional DCs (eBioscience). iDCs were cultured with T3 (5 nmol/L; Sigma) or LPS (100 ng/mL; *E coli* strain 0111:B4; Sigma) for 18 hours. Parallel cultures were maintained without stimuli as controls. T3 was prepared according to the manufacturer's recommended protocol. To rule out endotoxin contamination of the T3 preparation, we checked endotoxin content that raised levels lower than 0.03 IU/mL (limit of detection) by the Limulus amebocyte lysate assay (Sigma).

Flow cytometric analysis of DC phenotype

DCs were washed with PBS supplemented with 2% (vol/vol) FCS and resuspended in 10% (vol/vol) FCS in PBS. Cells were then incubated for 30 minutes at 4°C with the following

fluorochrome-conjugated mAbs: FITC-anti-CD11c, phycoerythrin (PE)-anti-IA/IE (MHC-II), PE-anti-CD40, PE-anti-CD80, PE-anti-CD86, and PECy7-anti-CCR7 (BD Biosciences PharMingen). Cells were processed and analyzed in a FACS Canto II flow cytometer (BD Biosciences) using FlowJo software (Tree Star).

Cytokine determination

Intracellular cytokine detection was assessed by flow cytometry as described (15) using PE-conjugated anti-IL12 mAb (BD Biosciences). IL10 and IFNγ detection was performed in culture supernatants using standard capture ELISA (BD Biosciences; ref. 15).

Allogeneic T-cell cultures

Allogeneic T-cell cultures were performed as described (15). Briefly, allogeneic splenocytes (BALB/c, 1×10^5 cells/well, responder cells) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 μmol/L) at 37°C for 15 minutes. Labeled cells were incubated for 3 days with irradiated DCs (1:15, DC:splenocytes). Cells were analyzed by FACS gating on CFSE-labeled cells to assess allogeneic T-cell proliferation induced by DCs.

Protein extraction and Western blotting

Protein extracts were obtained as described (15). Phospho-Akt (5473), Bcl-2 (50E3), and GAPDH (D16H11) rabbit monoclonal Abs were from Cell Signaling Technology, Inc. Western blot was performed as described (17).

In vivo cytotoxic assays

Cytotoxic assays were conducted as described (19). Mice were i.v. immunized with 5×10^6 DCs incubated for 18 hours with: (i) PBS (control), (ii) ovalbumin (OVA;100 μg/mL; Worthington), (iii) OVA+LPS (100 ng/mL), or (iv) OVA+T3 (5 nmol/L). Spleen cells from syngeneic mice were labeled with either 3 μmol/L CFSE ($CFSE^{high}$) or 0.5 μmol/L CFSE ($CFSE^{low}$) in Hank's Balanced Salt Solution and then washed in Hank's Balanced Salt Solution 10% FCS. $CFSE^{high}$ cells were pulsed with $OVA_{257–264}$ peptide (10 ng/mL; Sigma) for 30 minutes at 37°C. $CFSE^{low}$ cells were not pulsed and served as an internal control. On day 7 after immunization, mice were injected i.v. with a mixture of 3×10^6 $CFSE^{low}$ and 3×10^6 $CFSE^{high}$ cells. Spleen cells were analyzed by flow cytometry 24 hours later.

Antigen cross-presentation assay

This assay was conducted as described (20). Briefly, DCs were incubated for 4 hours with (i) PBS (control), (ii) OVA (100 μg/mL), (iii) $OVA_{257–264}$ (10 ng/mL), or (iv) OVA+T3 (5 nmol/L). Besides, B3Z T hybridoma cells [which express LacZ upon binding of the T-cell receptor (TCR) to $OVA_{257–264}$/Kb complex] were placed at different effector/target cell ratios. After coincubation of DCs with B3Z T hybridoma cells overnight, they were lysed by addition of 125 μL of a solution containing 5 mmol/L o-nitrophenyl-p-D-galactoside (ONPG) in PBS/0.5% NP-40. After 4-hour incubation at 37°C, the amount of LacZ enzyme was quantified by the hydrolysis of ONPG, measuring optical density (OD) at 415 nm.

Cell death assays

After DC treatment with dexamethasone (10 μmol/L; Sigma) and/or T3 (5 nmol/L) for 18 hours, cell death was evaluated by flow cytometry using the FITC-Annexin V binding assay and 7-aminoactinomycin D (7-AAD; BD Biosciences) as described (17).

DC migration *in vivo*

Control and T3-stimulated DCs labeled with CFSE (5 μmol/L) were injected s.c. into mice. Lymph node DC-CFSE$^+$ cells were analyzed by flow cytometry 24, 48, and 72 hours later.

RNA extraction and reverse transcription/qPCR

Total RNA isolation and cDNA synthesis were performed as described (16). qPCR analysis was carried out using an ABI Prism 7500 detection system (Applied Biosystems) and SYBR Green chemistry as described (16). Gene-specific primer sets were as follows: 5′-GGCTCTCCTTGTCATTTTCCAG-3′ (CCR7 forward), 5′-AATACATGAGAGGCAGGAACCAG-3′ (CCR7 reverse), 5′-GGCACCACACTTTCTACAATG-3′ (β-actin forward) and 5′-TGGCTGGGGTGTTGAAGGT-3′ (β-actin reverse). To determine relative changes in CCR7 gene expression, the $2^{-\Delta\Delta Ct}$ method was used and normalized against the housekeeping gene β-actin as internal control. All primers were from Sigma-Genosys.

B16-OVA tumor model

The syngeneic melanoma B16.F1-OVA line (gift from Dr. Dellabona, Fondazione Centro San Raffaele del Monte Tabor-San Raffaele Scientific Institute, Milan, Italy) was used (21). Cells were passaged every 2 to 3 days, and growing cultures were used to generate tumors. For tumor induction, 2×10^4 B16.F1-OVA cells were administered s.c. in 250 μL PBS in the left flank of C57BL/6 mice. Animals were monitored for tumor growth by palpation, and tumor size was measured every 2 to 3 days with a caliper (tumor volume = $L \times W^2/2$, where L = length and W = width). Animal survival was defined as the time between tumor cell inoculation and the day of sacrifice (tumor diameter, 20 mm; ref. 22).

DC-based immunotherapeutic protocol

DCs were treated with OVA (100 μg/mL, DC + OVA), OVA and T3 (5 nmol/L, DC+OVA +T3), or PBS (control). Treated DCs (1.5×10^6) were injected s.c. on the contralateral flank of tumor-bearing mice at days 1, 3, 5, and 8 after tumor cell (B16.F1-OVA) inoculation.

Analysis of tumor-infiltrating cells

Tumors were removed and single-cell suspensions were prepared by enzymatic digestion and submitted to Ficoll–Hypaque (Sigma) gradient centrifugation as described (23). Immune cell populations (CD8, CD4, and NK) were determined by flow cytometry by incubating with PECy5-anti-CD4, PE-anti-CD8, and FITC-anti-NK1.1 mAbs (BD Biosciences). Cells were then analyzed in a FACS Canto II flow cytometer (BD Biosciences) using FlowJo software (Tree Star).

Cytokine production

Ten days after the last DC transfer, splenocytes were stimulated with $OVA_{257–264}$. After 72 hours, IFNγ secreted by tumor-specific T cells was determined by ELISA (BD Biosciences). Intracellular IFNγ was determined by flow cytometry (15).

Statistical analysis

Analysis of intergroup differences was conducted by one-way ANOVA, followed by the Student-Newman-Keuls test. Survival differences and rates of tumor establishment were compared using the Gehan-Breslow-Wilcoxon test. *P* values less than 0.05 were considered statistically significant. All experiments were performed at least in triplicate.

Results

Binding of T3 to TRβ promotes DC maturation and function *in vivo*

To elucidate the pathophysiologic relevance of the $TR\beta_1$ *in vivo*, we evaluated the effects of T3 action in the maturation and function of bone marrow-derived DCs from TRβPV mice. For this purpose, DCs from WT (DC^{WT}) and homozygous TRβPV (DC^{PV}) mice were exposed to T3 and analyzed for the expression of MHC-II and costimulatory molecules, as well as for IL12 and IL10 production. Whereas T3 promoted maturation of DC^{WT} (15), as revealed by increased expression of MHC-II, CD40, CD80, and CD86 on the surface of these cells, DC^{PV} exhibited a prominent immature phenotype characterized by marked expression of CD11c, and lower expression of maturation markers (Fig. 1A, left and right plots). Moreover, the immature phenotype of DC^{PV} was associated with a reduced DC functionality as T3 could not increase the frequency of IL12-producing DC^{PV} when compared with T3-stimulated DC^{WT} (Fig. 1B).

The prominent immature phenotype of T3-conditoned DC^{PV} prompted us to investigate the T-cell allostimulatory capacity of these cells. In contrast with T3-matured DC^{WT} (15), T3-conditioned DC^{PV} did not increase the proliferation of BALB/c (H-2d) splenocytes (Fig. 1C). The poor allostimulatory capacity was also reflected by diminished frequency of IFNγ-producing T cells and reduced IFNγ secretion (Fig. 1D and E, respectively). These results were comparable with those observed in DCs isolated from lymph nodes and spleens from WT and TRβPV mice (Fig. 1F and G, respectively). In addition, lymph node cells but not splenocytes from TRβPV mice showed higher IL10 secretion when compared with WT mice. Interestingly, whereas T3 induced $Akt^{Ser-473}$ phosphorylation after 30 minutes in T3-stimulated DC^{WT} (16), this effect was abrogated in DC^{PV} (Fig. 1H). Taken together, these results highlight the critical role of intact TRβ expression in the control of DC signaling and function *in vivo*.

T3 instructs DCs to stimulate cytotoxic T-cell responses

We previously reported the ability of T3 to induce a mature DC phenotype capable of skewing the balance toward a dominant Th1 profile (15), which might influence the development of cytotoxic T-cell responses. However, T3 failed to exert this effect in DCs from TRβPV mice (Fig. 1), a mutation linked to cancer development (7). Taken together, these evidences prompted us to ascertain the role of T3 in cancer by assessing the ability of

T3-stimulated DCs to modulate antigen-specific T-cell cytotoxicity in *in vivo* cytotoxicity assays.

Control and OVA-stimulated DCs failed to mount a robust cytotoxic-specific response (similar $CFSE^{high}/CFSE^{low}$ profile, mean cytotoxic activity: 37.95%; Fig. 2A and B). However, immunization with OVA-pulsed T3-stimulated DCs resulted in activation of OVA-specific cytotoxic responses capable of clearing $OVA_{257–264}$ peptide-coated splenocytes (mean cytotoxic activity: 87.41%) similar to the positive control group (LPS, mean cytotoxic activity: 100.00%; ref. 24). These results show the capacity of T3-matured DCs to trigger antigen-specific cytotoxic responses *in vivo*.

As antigen cross-presentation is essential for mounting cytotoxic T-cell responses against tumors (9), the implications of the T3-TRβ signaling in this process were assessed. We used B3Z T hybridoma cells, which express LacZ upon binding of TCR recognizing the OVA peptide presented in the context of MHC I. As shown in Fig. 2C, OVA-pulsed T3-stimulated DCs significantly increased β-Gal activity when compared with OVA-pulsed or unpulsed DCs at all B3Z:DC ratios analyzed. This T3-mediated effect was comparable with that induced by $OVA_{257–264}$ (a positive control), suggesting modulation of cross-presentation by T3 signaling.

T3 modulates DC viability

Apoptosis of DCs is a key event in the control of tolerance versus immunity and has been documented in several types of solid and blood cancers (25–27). As induction of apoptosis favors the establishment of immunosuppressive microenvironments characterized by the differentiation of T regulatory cells and impairment of DC functionality (28), we evaluated the effect of T3 on DC survival. Cell death was assessed in DCs treated or not with T3 or dexamethasone, a typical proapoptotic stimulus (29). As shown in Fig. 3A and B, T3 decreased the number of apoptotic (Annexin $V^+/7\text{-}AAD^-$) and necrotic (Annexin $V^+/7\text{-}AAD^+$) cells compared with control DCs. Moreover, T3 counteracted the known proapoptotic effect of dexamethasone in DCs. After 30 minutes of stimulation, T3 increased the expression of Bcl-2 (30), an effect that persisted even after 18 hours (Fig. 3C). Interestingly, T3-induced Bcl-2 upregulation correlated with increased DC survival (Fig. 3A and B). Thus, exposure to T3 increases DC resistance to apoptosis, an effect that may extend DC lifespan.

T3 enhances the migratory capacity of DCs to lymph nodes

To elicit a primary immune response, DCs migrate to lymph nodes where they reach T cells for activation (31). We evaluated the ability of T3 to induce DC migration to lymph nodes by tracking CFSE-labeled DCs ($CFSE^+/CD11c^+$) by flow cytometry. Although the percentage of double-positive DCs in lymph nodes was low when they were not exposed to T3, it was significantly enhanced in T3-treated DCs (Fig. 4A). In turn, a time-course experiment showed that DC migration was increased in response to T3 after 72 hours of injection, but it was not different at 24 and 48 hours (DC-T3 vs. DC; Fig. 4B).

Migration of DCs to secondary lymphoid organs and tissues relies on a cascade of discrete events, including regulation of chemokines and their specific receptors (32). As CCR7 has an essential role in DC homing to lymph nodes (33), CCR7 expression was determined in

T3-stimulated DCs. Exposure to T3 resulted in a significant increase in CCR7 protein (Fig. 4C and D) and mRNA (Fig. 4E) to levels comparable with positive control (LPS; ref. 34).

T3-stimulated DCs instruct the development of $CD8^+$ T-cell-mediated responses

To further assess the role of T3-stimulated DCs on tumor immunity, we investigated whether T3 may enhance the therapeutic potential of DC vaccines *in vivo*. We established a mouse model of B16-OVA melanoma (B16 cells in which OVA was stably expressed to allow monitoring of antigen-specific responses). We treated mice with T3-stimulated DCs in the presence of tumor antigen (OVA; Fig. 5A). Mice immunized with T3-stimulated DCs plus OVA showed a significant delay in tumor onset (Fig. 5B), as well as a substantial decrease in tumor size measured at day 27 (Fig. 5C). In turn, s.c. administration of T3-stimulated DCs (+OVA+T3) resulted in prolonged mice survival compared with DC+OVA (Fig. 5D): 60% DC+OVA+T3 versus 20% DC+OVA at 40 days after tumor inoculation. Of note, all saline-treated mice died within 40 days after the B16-OVA implantation. Accordingly, there was a higher number of tumor-free mice when they were injected with T3-pulsed DCs (+OVA+T3) compared with mice receiving untreated DCs (+OVA) or controls (Fig. 5E).

T3-stimulated DCs increase the number of tumor-infiltrating $CD8^+$ T cells and augment IFNγ-producing antigen-specific immune responses

To further investigate the effect of T3 on DC-mediated antitumor responses, we evaluated the number of tumor-infiltrating T cells by flow cytometry. Flow cytometric analysis revealed an increased number of infiltrating intratumoral $CD8^+$ T cells in mice receiving DC +OVA+T3 compared with DC+OVA (Fig. 6A). However, we could find no significant differences when comparing $CD4^+$ T cells (Fig. 6A) and NK cells (data not shown) between the experimental groups analyzed. Thus, T3 endows DCs with the ability to augment a cytotoxic T-cell response that restrains tumor growth *in vivo*.

As T3-conditioned DCs induced prolonged survival and intratumoral $CD8^+$ T-cell infiltration in mice bearing B16-OVA tumors, we aimed at evaluating whether a systemic tumor-specific immune response was induced by T3-stimulated DCs. Secretion of IFNγ was significantly higher in splenic T cells derived from mice injected with DC+OVA+T3 compared with mice treated with DC+OVA in the absence of T3 (Fig. 6B, white boxes). Notably, when T cells from mice treated with T3-stimulated DCs were restimulated with $OVA_{257–264}$ (Fig. 6B, black boxes), a 3-fold increase in IFNγ secretion was registered (DC +OVA+T3 vs. DC+OVA), indicating an antigen-specific antitumor T-cell response. By intracellular staining, we found that IFNγ production was mainly restricted to $CD8^+$ T cells (Fig. 6C). Therefore, DCs exposed to T3 increase the frequency of IFNγ-secreting $CD8^+$ T cells and bolster antigen-specific antitumor T-cell responses.

Discussion

Biologic signals that control DC maturation and function can ultimately tailor adaptive immune responses (8). Our study highlights an alternative mechanism by which T3 contributes to cancer progression by acting at the DC level and bolstering $CD8^+$ T-cell mediated antitumor responses.

In contrast with the reported effects induced by T3 on DCs from mice with intact $TR\beta_1$, the main TR isoform (15), T3 could not trigger maturation effects on DCs from homozygous TRβPV knock-in mice (DC^{PV}). Interestingly, T3-treated DC^{PV} showed a deficient T-cell allostimulatory capacity and impaired Akt activation when compared with DC^{WT} (16). These findings highlight a critical role of intact TRβ expression *in vivo* in the control of DC signaling and function as previously shown using small interfering RNA silencing strategies (16). The well-known association of TRβPV with cancer development (7), together with our previous observations indicating the promotion of a DC1 mature phenotype by T3 (15), suggested that manipulation of the T3-TRβ1 system on DCs could favor an IFNγ-mediated cytotoxic T-cell response and bolster antitumor immunotherapeutic strategies.

DCs share unique features and plasticity that make them an ideal choice for antitumor vaccines (35). DC-based vaccination strategies effectively evoke tumor-specific cytotoxic T-cell responses, which contribute to restrain tumor progression either alone or in combination with other immunotherapeutic approaches, including blockade of negative regulatory checkpoints such as CTLA-4 or PD-1/PD-L1 (36). However, the overarching goal of cancer vaccinologists is to elicit long-lasting tumor-specific $CD8^+$ T cell-mediated responses that are sufficiently robust to evoke durable tumor regression and/or eradication. In particular, cross-presentation of antigen is essential for induction of effective cytotoxic T-cell responses against tumors (37). Our results demonstrate that T3 potentiates DC function at multiple levels, including DC survival, migration, and allostimulatory T-cell capacity. Moreover, it instructs DCs to bolster antigen-specific cytotoxic responses *in vivo* and antigen cross-presentation *in vitro*. These findings further support the *in vivo* role of T3 in cancer biology. Further studies are in progress to elucidate the impact of regulatory T-cell responses elicited by T3-conditioned DCs in tumor and inflammatory microenvironments.

In DC-based cancer immunotherapies, DCs are usually isolated *in vitro* and loaded with tumor antigen. These antigen-bearing DCs are then injected back into patients, migrate into lymphoid tissues, and activate antigen-specific T cells for eliminating tumor cells. However, preservation of DC survival is a current challenge (35). DC's short lifespan probably evolved to prevent autoimmunity, but in the context of immunotherapy, short-lived, *ex vivo*-modified DC vaccines will likely undergo apoptosis before encountering and optimally activating tumor antigen-reactive T-cell clones. Interestingly, it has been demonstrated that immunization of mice with DCs infected with a Bcl-xL-encoding adenovirus effectively generated more potent antitumor immunity (38) and modulated Akt activation, which is a critical regulator of DC lifespan (12). Our findings demonstrate the ability of T3 to increase DC survival in accordance with our previous report, indicating an increase in Akt phosphorylation in T3-stimulated DCs (16). These results together with the modulation of Bcl-2 expression in T3-conditioned DCs emphasize the potential role of T3 treatment *ex vivo* as a powerful tool to increase the effectiveness of DC-based vaccines.

CCR7 is a chemokine receptor that drives DC migration to lymph nodes, inducing an increase in lymph node cellularity even before the onset of T-cell proliferation (33, 39). It has been reported that DC migration could be enhanced by preinjection of inflammatory cytokines that augment the expression of the CCR7 ligand, CCL21 (40). Here, we found that exposure of DCs to T3 increases CCR7 expression and favors DC migration to lymph nodes,

a critical event for T-cell encounter. Interestingly, CCR7 expression has been shown to extend DC survival through a mechanism involving Akt activation (41). This report is in line with our findings showing sustained Akt activation, increased CCR7 expression, and extension of DC lifespan induced by T3.

The influence of T3 in DC function and maturation prompted us to investigate the impact of T3-TRβ complex in DC-mediated T-cell activation and the development of antitumor responses in a mouse B16-OVA tumor model (21). Vaccination with T3-activated DCs in the presence of a tumor antigen (OVA) increased the percentage of tumor-free mice, delayed the kinetics of tumor growth, and improved mice survival. This vaccination protocol resulted in prominent infiltration of $CD8^+$ T cells within the tumor. These findings are of particular interest, as adoptive transfer of tumor-infiltrating lymphocytes leads to potent antitumor responses, cancer regression, and prolonged patient survival in a variety of tumor types (42, 43). Interestingly, we found that T cells isolated from tumor-bearing mice treated with T3-conditioned DCs displayed heightened IFNγ secretion and sustained $CD8^+$ T-cell responses. In agreement, therapeutic administration of DCs pulsed *in vitro* with the heat shock protein 70 in the presence of a cyclooxygenase-2 inhibitor, delayed the progression of B16 tumors in mice through mechanisms involving expansion of IFNγ-producing $CD8^+$ T cells (44). Noteworthy, our results were achieved taking advantage of OVA as a tumor antigen of B16-OVA. However, future research of the effect of T3-treated DCs in the presence of recombinant native-specific tumor peptides should be conducted in B16 as well as in other tumor models.

The majority of clinical trials involving DC-based vaccination uses a cocktail of proinflammatory cytokines, including TNF, IL1β, IL6, and PGE_2 (45). Moreover, other authors explored the use of CD40L (46) and Toll-like receptor agonists that differentially stimulate systemic and local innate immune responses (47). Taken together, our previous (15, 16) and current findings propose the emergence of T3 as an adjuvant alternative that educates DCs for stimulating antigen-specific effective T-cell responses in cancer settings. Moreover, given its physiologic origin, T3 may exhibit advantages over other artificial adjuvants, which could display higher toxicity.

Importantly, our study highlights an alternative mechanism by which thyroid hormone influence tumor growth inhibition in addition to other tumor suppressor roles of $TR\beta_1$ (48). In this sense, TRβPV mice develop follicular thyroid carcinoma and mutated TRs associate with several human cancers, including liver, kidney, pituitary, and thyroid tumors (7). On the other hand, diminished expression of TRβ mRNA has been implicated in the carcinogenesis of papillary thyroid carcinomas, kidney, and breast cancers (48). Hence, reduced DC immunogenicity documented in TRβPV mice and augmented cytotoxic T-cell responses triggered by T3-conditioned DCs may also contribute to tumor development and progression observed under TRβ deficiency. In contrast, other studies support the concept that WT TRs can enhance carcinogenesis, suggesting that TRs could play a dual role in carcinogenesis (49). Because TR actions are complex, tissue-restricted and stage-specific, aberrant expression of the various TR isoforms might have divergent roles in diverse tumor types and/or at different stages of tumor development (49). Our findings suggest a novel mechanism by which the T3-TRβ complex influences antitumor response by bolstering DC-

mediated T-cell activation during tumor growth. Interestingly, although the experiments shown here were performed at T3 physiologic concentrations, further work should be aimed at elucidating the impact of different thyroid states (hypothyroid, euthyroid, or hyperthyroid) in DC functionality *in vivo* and antitumor responses.

In summary, our findings emphasize the physiologic roles of the thyroid hormone in the development of antitumor response and highlight the use of T3-conditioned DCs as an alternative approach to potentiate T-cell–mediated tumor immunity.

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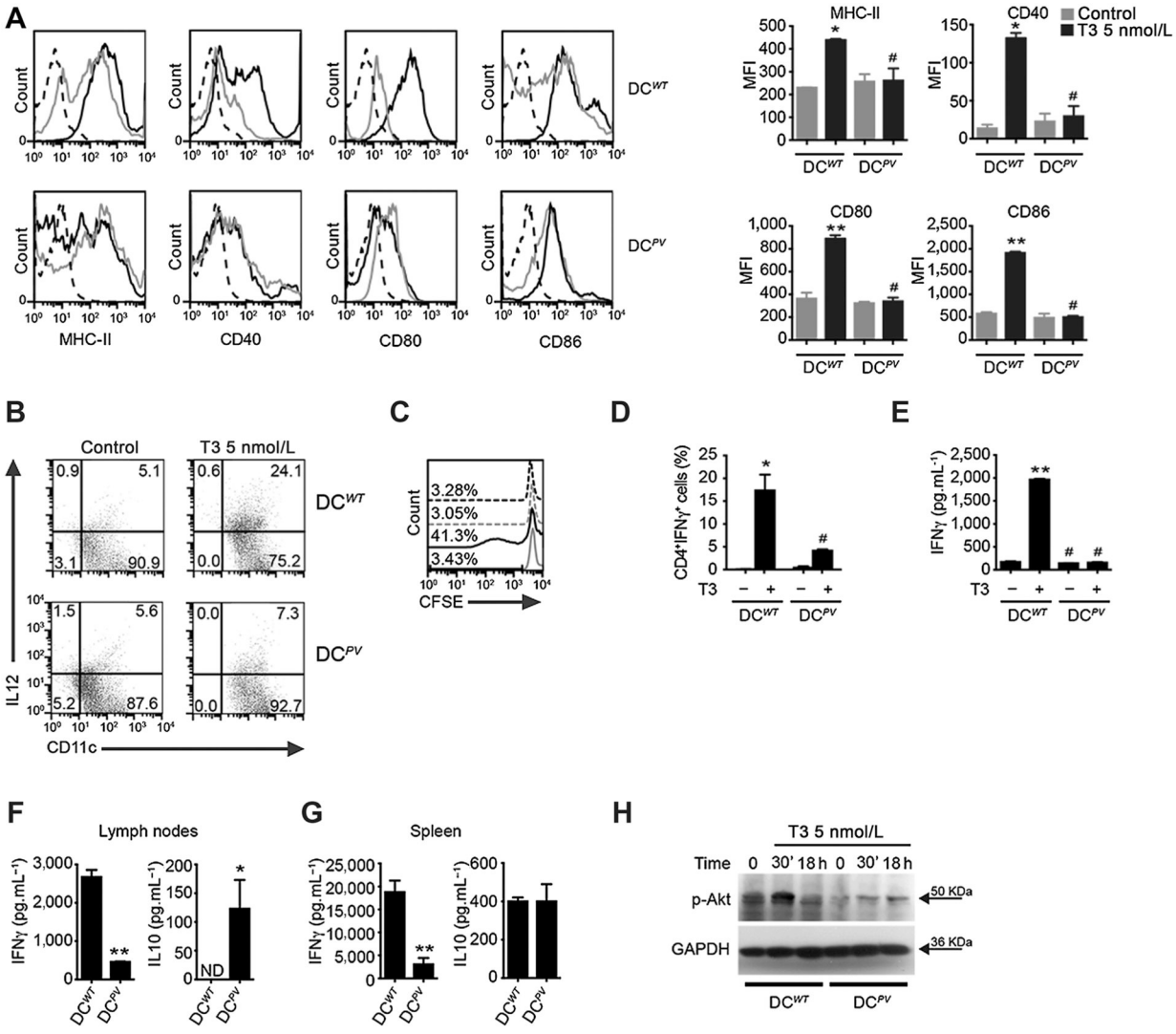

Figure 1.

Role of TRβ signaling in T3-induced DC maturation and function *in vivo*. DC^{WT} and DC^{PV} were treated with T3 or left untreated and analyzed as described. A, flow cytometry analysis of phenotypic markers. Histograms are gated on $CD11c^+$ cells (left plot). Dotted histograms, nonspecific binding determined with isotype-matched control antibodies (IC); gray histograms, control DC phenotypic markers; black histograms, T3-stimulated DC phenotypic markers. Results are expressed as mean fluorescence intensity (MFI) within the $CD11c^+$ population (right plot). B, intracytoplasmic detection of IL12p70. Values are given as the percentage of total $CD11c^+$ IL12-producing cells. C–E, allostimulatory capacity of DCs. C, proliferation of allogeneic splenic T cells was determined by CFSE dilution. Continuous and dotted lines represent proliferation of allogeneic splenic T cells cultured with DC^{WT} and DC^{PV}, respectively. D and E, IFNγ production was evaluated by flow cytometry and ELISA, respectively. F and G, IFNγ and IL10 production in allogeneic T cell cultures were measured by ELISA. H, Akt phosphorylation in DCs was evaluated by Western blot. Data are expressed as mean ± SD and are from a representative of three

experiments. *, $P < 0.05$; **, $P < 0.001$ vs. control DC^{WT}; #, $P < 0.05$ vs. T3-stimulated DC^{WT}.

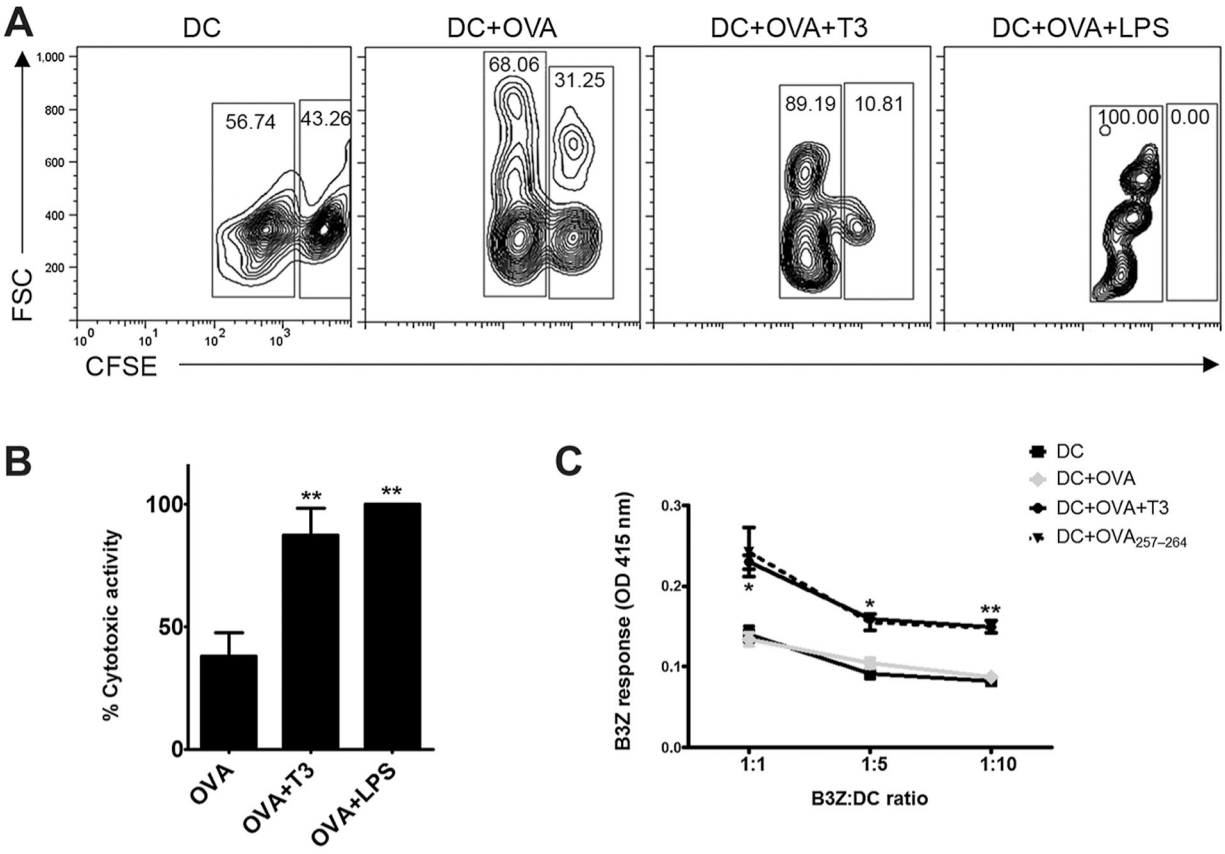

Figure 2.

T3 increases the ability of DCs to stimulate antigen-specific cytotoxic T-cell responses and antigen cross-presentation. *In vivo* cytotoxicity (A and B) and *in vitro* antigen cross-presentation (C) assays were performed as described. A, density graphs represent the amount of CFSE-labeled cells. Number in each plot indicates the percentage of $CFSE^{low}$ and $CFSE^{high}$ cells. B, cytotoxic activity was determined by calculating the percentage of specific lysis of peptide-pulsed $CFSE^{high}$ cells following the formula: 100–(percentage of peptide pulsed/percentage of unpulsed in immunized mice)/(percentage of peptide pulsed/percentage of unpulsed in control mice) × 100). C, OD at 415 nm in each experimental group is shown. Data are expressed as mean ± SD and are from a representative of three experiments. *, $P < 0.01$; **, $P < 0.001$ vs. DC+OVA.

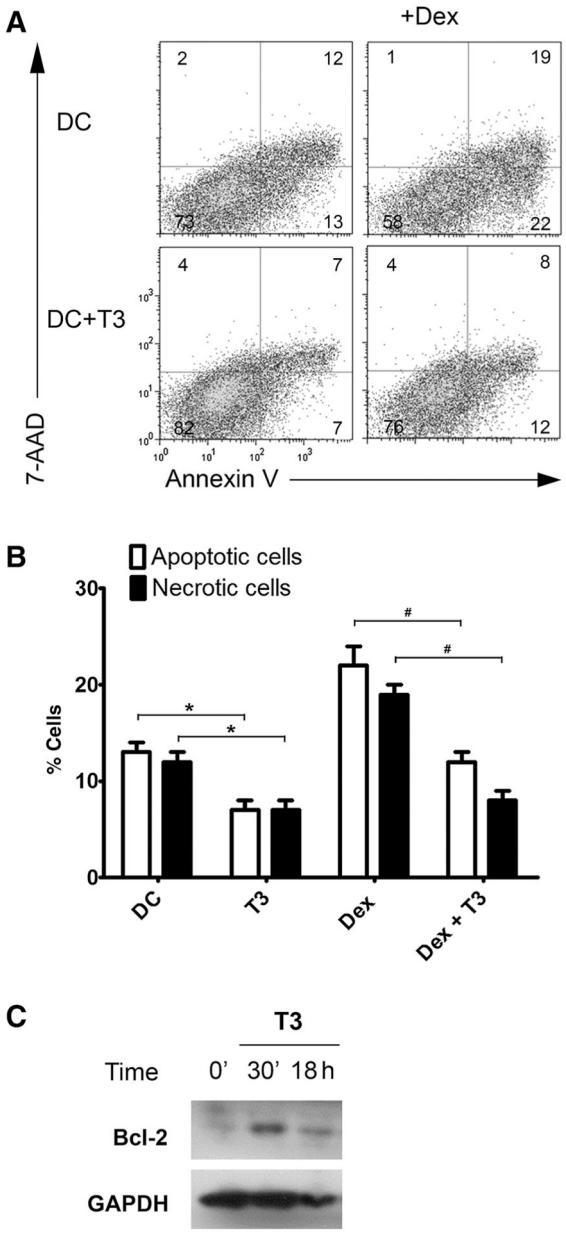

Figure 3.

T3 increases DC viability. iDCs were treated with T3, Dex, or left untreated. A and B, DC viability was assessed as described. Cells were gated on $CD11c^+$ cells, and Annexin V and 7-AAD were analyzed. A, numbers represent the percentage of cells in each quadrant. Cells in early phases of apoptosis were Annexin $V^+/7\text{-}AAD^-$ and late-stage apoptotic and necrotic cells were Annexin $V^+/7\text{-}AAD^+$. B, values are given as the percentage of apoptotic and necrotic cells within total $CD11c^+$ cells. Data are expressed as mean ± SD and are from a representative of three experiments; *, $P < 0.001$ vs. apoptotic and necrotic control DCs, respectively; $^{\#}$, $P < 0.001$ vs. apoptotic and necrotic Dex-DCs, respectively. C, analysis of Bcl-2 by Western blot. Bottom, same blot probed for GADPH to check for equal loading. Representative of three experiments.

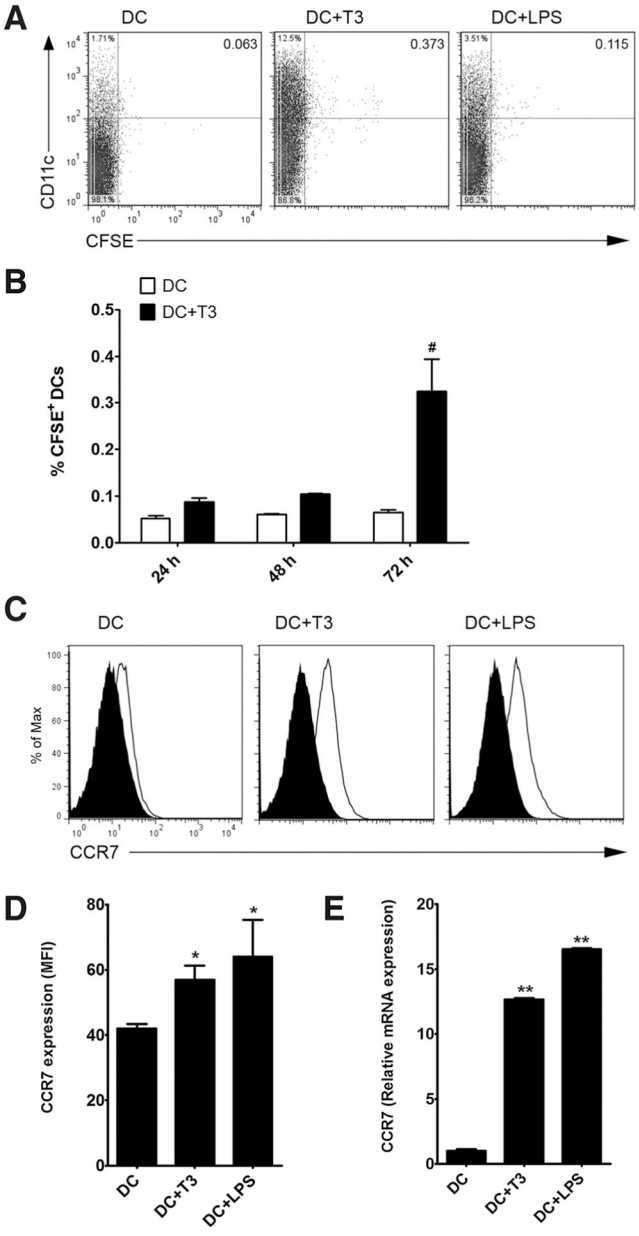

Figure 4.

T3 increases the migratory capacity of DCs to lymph nodes. iDCs were treated with T3, LPS, or left untreated. A and B, migration of DCs *in vivo* was determined as described in Materials and Methods. A, cell migration to lymph nodes after 72 hours. Numbers represent the percentage of cells in each quadrant. B, time-course study of migration of $CD11c^+/CFSE^+$ cells to lymph nodes (24, 48, and 72 hours). Values are given as the percentage of $CD11c^+/CFSE^+$ cells of total lymph nodes cells. C–E, CCR7 expression was analyzed by flow cytometry (C and D) and qPCR (E). C, representative histograms of three independent experiments are gated on $CD11c^+$ cells. Black histograms, nonspecific binding determined with isotype-matched control antibodies; white histograms, CCR7. D, results are expressed as mean fluorescence intensity (MFI) within the $CD11c^+$ population. Data are expressed as

mean ± SD and are from a representative of three experiments; #, $P < 0.05$ vs. DC 72 h and DC+T3 48 h; *, $P < 0.05$; **, $P < 0.001$ vs. DC.

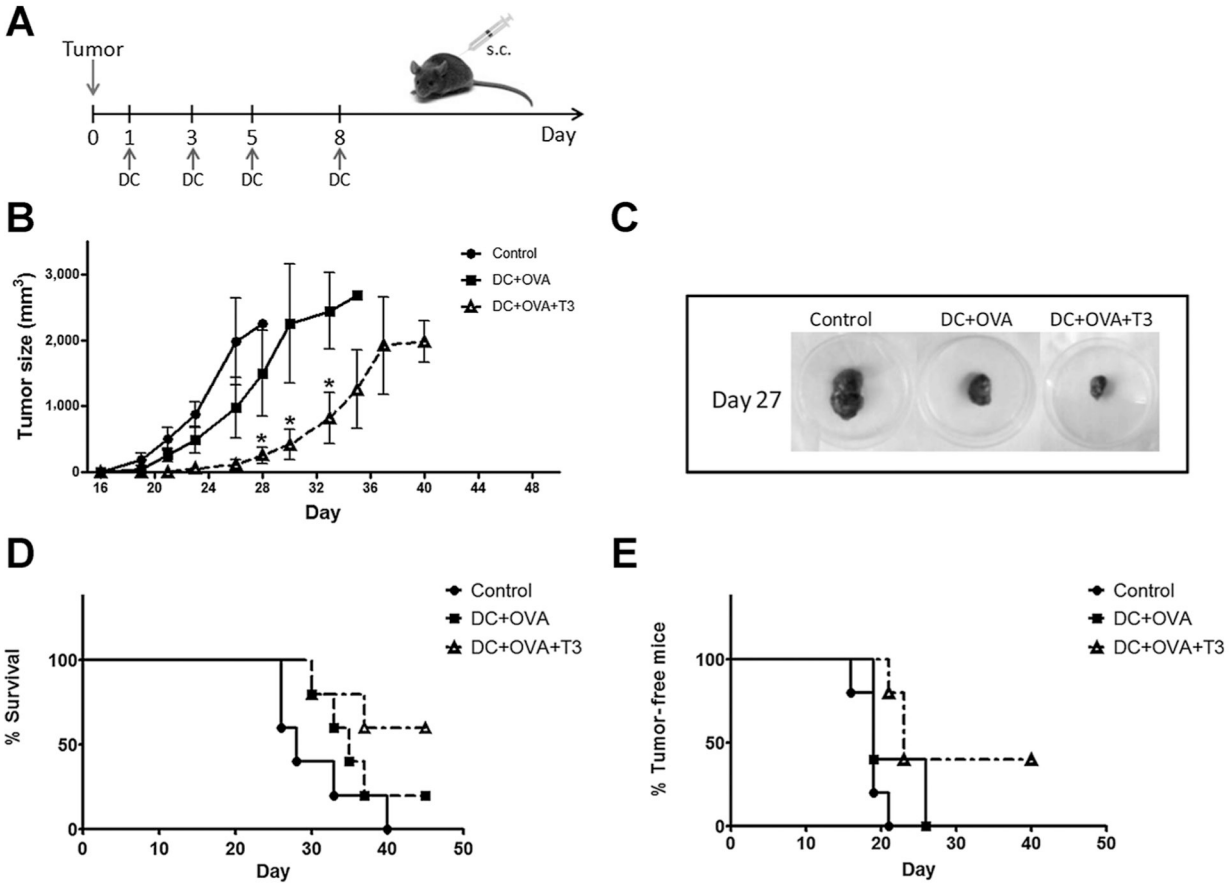

Figure 5.

T3-stimulated DCs in the presence of tumor antigen induce potent antitumor T-cell responses. A, schematic representation of the treatment of B16-OVA melanoma with control, OVA (DC+OVA), OVA, and T3 (DC+OVA+T3) DCs. B, tumor growth kinetics. Data are expressed as mean ± SD; *, $P < 0.05$ vs. DC+OVA. C, photographs of tumors removed on day 27. D and E, Kaplan—Meier analysis showing the percentages of surviving mice (D) and tumor-free mice (E) of each experimental group. Results are from a representative of three experiments ($n = 5$).

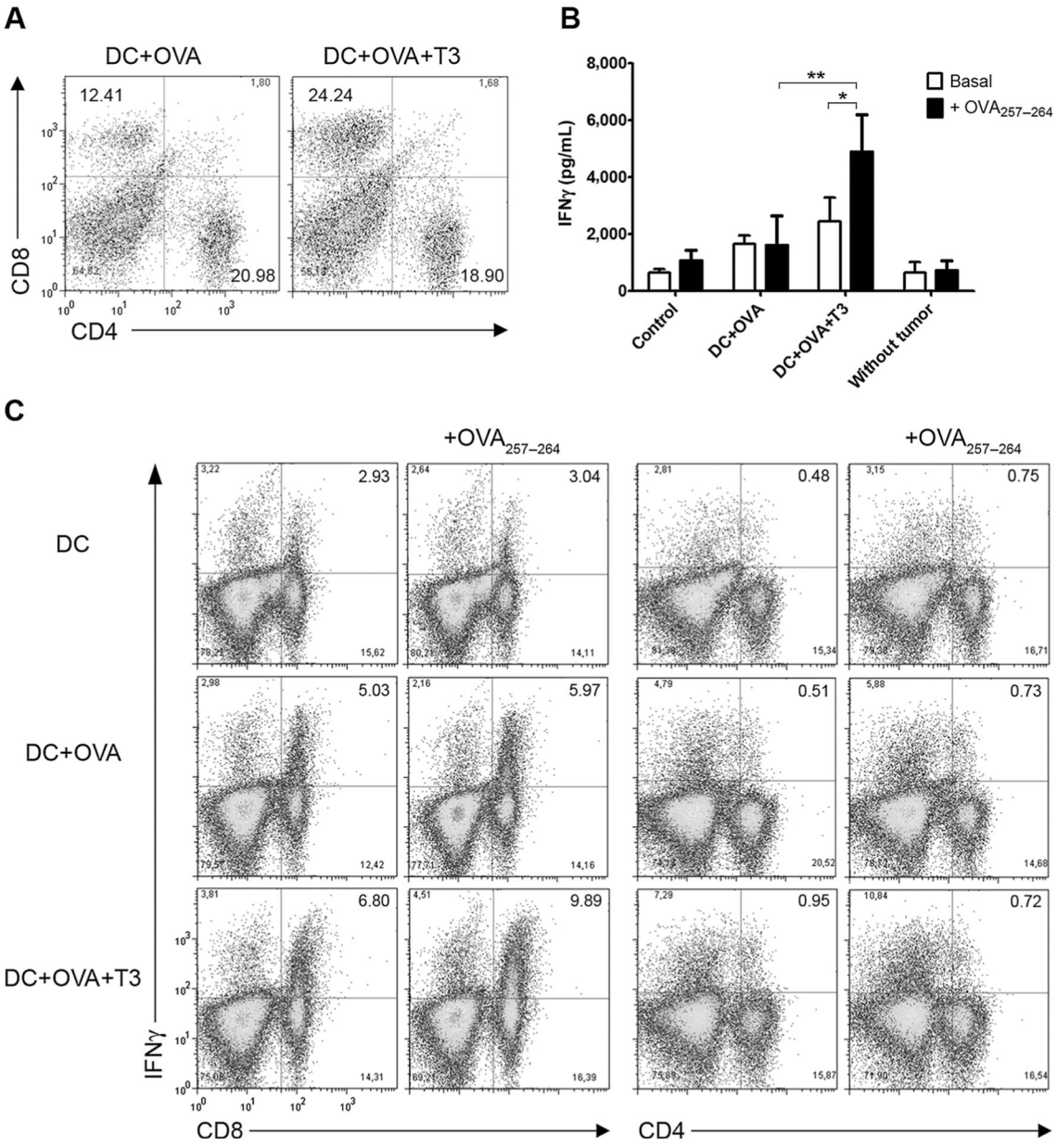

Figure 6.

Vaccination with T3-stimulated DCs augments the frequency of intratumoral $CD8^+$ T cells and increases IFNγ production. Animals were treated as described in Fig. 5A, and sacrificed on day 27. A, tumor-infiltrating cells were determined by flow cytometry. Numbers represent the percentage of cells in each quadrant. B, IFNγ production by splenic T cells from tumor-bearing mice determined by ELISA. C, the frequency of IFNγ-secreting $CD4^+$ and $CD8^+$ splenic T cells was determined by flow cytometry. The numbers in each quadrant represent the percentage of total cells expressing CD4 or CD8 and IFNγ. Data are expressed as mean ± SD. Results are from a representative of three experiments ($n = 5$). *, $P < 0.001$ vs. basal DC+OVA+T3; **, $P < 0.001$ vs. stimulated DC+OVA.