Dissecting thyroid hormone transport and metabolism in dendritic cells

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Abstract

We reported thyroid hormone (TH) receptor expression in murine dendritic cells (DCs) and 3,5,3′-triiodothyronine (T_3)-dependent stimulation of DC maturation and ability to develop a Th1-type adaptive response. Moreover, an increased DC capacity to promote antigen-specific cytotoxic T-cell activity, exploited in a DC-based antitumor vaccination protocol, was revealed. However, putative effects of the main circulating TH, L-thyroxine (T_4) and the mechanisms of TH transport and metabolism at DC level, crucial events for TH action at target cell level, were not known. Herein, we show that T_4 did not reproduce those registered T_3-dependent effects, finding that may reflect a homoeostatic control to prevent unspecific systemic activation of DCs. Besides, DCs express MCT10 and LAT2 TH transporters, and these cells mainly transport T_3 with a favored involvement of MCT10 as its inhibition almost prevented T_3 saturable uptake mechanism and reduced T_3-induced IL-12 production. In turn, DCs express iodothyronine deiodonases type 2 and 3 (D2, D3) and exhibit both enzymatic activities with a prevalence towards TH inactivation. Moreover, T_3 increased MCT10 and LAT2 expression and T_3 efflux from DCs but not T_3 uptake, whereas it induced a robust induction of D3 with a parallel slight reduction in D2. These findings disclose pivotal events involved in the mechanism of action of THs on DCs, providing valuable tools for manipulating the immunogenic potential of these cells. Furthermore, they broaden the knowledge of the TH mechanism of action at the immune system network.

Introduction

Thyroid hormones (THs) are critical regulators of cellular differentiation, growth and metabolism. The thyroid secretes 100% of circulating thyroxine (T_4). However, it provides a low percentage of serum levels of the most physiologically active TH: 3,5,3′-triiodothyronine (T_3), the major part of which is derived from peripheral 5′ deiodination of T_4 (Williams & Bassett 2011).

Cellular activity of THs is classified as genomic (nuclear) and nongenomic (initiated either in the

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cytoplasm or at the plasma membrane). However, the main mechanism of TH action requires the participation of T_3 and its nuclear receptors (TRs): TRα1, TRβ1, TRβ2, and TRβ3, whereas other non-T_3-binding isoforms are also expressed (Bernal *et al*. 2015). It is noteworthy that the action of TH requires the proper interplay among cellular TH transporters, TH deiodinases and TRs expression (Williams & Bassett 2011, Kwakkel *et al*. 2014).

The uptake–efflux of THs by target cells is facilitated by proteins with different specificities for iodothyronine transport. The main TH transporters include monocarboxylate transporters (MCT) 8 and 10, organic anion transporter polypeptides (OATP) 1C1 and large neutral amino acid transporters (LAT) 1 and 2. MCT8, MCT10 and LATs have higher affinity for T_3 uptake than T_4 (Bernal *et al*. 2015).

The concentrations of THs at cellular level are regulated by the activity of three selenoproteins termed type 1, 2 and 3 iodothyronine deiodinases (D1, 2 and 3 encoded by *Dio* 1, 2 and 3 genes). D1 and D2 catalyze the outer ring deiodination that converts T_4 to T_3, whereas D3 deiodinates the inner ring iodides, thus initiating the conversion of T_4 and T_3 in inactive metabolites. Therefore, D2 is considered an 'activating' enzyme, responsible for the peripheral production of 50–80% of the total body pool of T_3. Conversely, D3 restrains T_3 action in target cells. TH transporters and deiodinases exhibit a particular expression profile that is cellular and metabolic state specific, as well as characteristic in several pathological conditions (Williams & Bassett 2011, Kwakkel *et al*. 2014, Bernal *et al*. 2015).

The effects of THs on several immune cells and their functions have been reported (De Vito *et al*. 2011, 2012, Cremaschi *et al*. 2016). However, studies on the impact of THs on the initiation of adaptive immunity at the level of antigen-presenting cells (APCs) are just emerging. We provided the first evidence of the expression of TRs, mainly the β1 isoform, and demonstrated that T_3 contributes to the maturation of the main APC: dendritic cells (DCs). A Th1-lymphocyte T adaptive response induced by physiologic levels of T_3 was also revealed (Mascanfroni *et al*. 2008). Mechanistically, these effects involved the activation of Akt and NF-kB pathways (Mascanfroni *et al*. 2010) and were counteracted by glucocorticoids (Montesinos *et al*. 2012). Moreover, we recently revealed that T_3 endows DCs with enhanced ability to stimulate cytotoxic T-cell responses with implications in DC-based immunotherapy (Alamino *et al*. 2015). However, the effect of T_4 at DC level and the mechanisms of TH

entrance and metabolization in DCs were unknown and disclosed in this work. The results obtained unravel new insights into TH action at the initiation of the immune response and provide tools for the manipulation of the potential immunogenicity of DCs, with profound impact on immunotherapy. To the best of our knowledge, this is the first systematic report of both mechanisms fully dissected in a cell from the immune system.

Materials and methods

Materials

$[^{125}I]T_3$ and $[^{125}I]T_4$ were from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Nonradioactive iodothyronines and leucine were from Sigma. Materials were from cell culture and of molecular biology grade.

Mice

Female C57BL/6 mice (B6; H-2b) were obtained from Ezeiza Atomic Center (Argentina). Mice were maintained under specific pathogen-free conditions and used when they were aged 6–10 weeks. 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.

DCs preparation and culture

Immature bone marrow DCs (iDCs) were obtained as described (Mascanfroni *et al*. 2008). Briefly, bone marrow progenitors were collected from the femurs of 6- to 10-week-old female C57BL/6 mice and cultured in RPMI-1640 10% fetal calf serum (FCS) depleted of THs by treatment with resin AG-1-X8 (Bio-Rad Laboratories) in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) from supernatant of the J558 cell line and fed every 2 days. At day 10 of cell culture, >85% of the harvested cells expressed MHC class II, CD40, CD80 and CD11c, but not Gr-1. Immature DCs were cultured with T_3 (5 nM) for 18 h (that induce DC maturation: mDCs) or with T_4 at times and concentrations indicated. Parallel cultures were maintained without stimuli as controls. To rule out endotoxin contamination of T_3 and T_4 preparations, we checked the endotoxin content that raised levels lower than 0.03 IU/mL (limit of detection) by the Limulus amebocyte lysate assay.

Flow cytometric analysis

Intracellular IL-12 and CD11c detection by flow cytometry was performed as previously described (Alamino et al. 2015) using the following fluorochrome-conjugated monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-anti-CD11c and phycoerythrin (PE)-anti-IL-12 (BD Biosciences, San Jose, CA, USA).

Cytokine determination

IL-12p70 detection was performed in cell culture supernatants using standard capture enzyme-linked immunosorbent assays (ELISA), as reported (Montesinos et al. 2012). Briefly, coating antibody included a rat anti-mouse IL-12p70 mAb (clone C15.6, BD Biosciences). Detection antibody included biotinylated rat anti-mouse IL-12p70 mAb (clone C17.8, BD Biosciences). Streptavidin-horseradish peroxidase and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma) were used as enzyme and substrate, respectively.

Reverse transcription (RT) and polymerase chain reaction (PCR)

Cells were homogenized with TRIzol (Sigma), and RNA extraction was performed according to the manufacturer's recommended protocol. mRNA was reverse transcribed and amplified by PCR essentially as described (Mascanfroni et al. 2008). Gene-specific primer sets to amplify TH transporters (Mct8, Mct10, Oatp1c1, Lat1 and Lat2) and iodothyronine deiodinases (Dio 1, 2 and 3) mRNAs were from Sigma (Buenos Aires, Argentina, Table 1). Mct8, Oatp1c1, Lat1, Lat2 and Dio1 primers were designed to distinguish cDNA and genomic DNA/pseudogenes linking the sequences of 2 exons and excluding an intron (Kreuzer & Neece 1999). For Dio 2 and 3 mRNA, as the primers were designed in an exon region, genomic DNA contamination was excluded by performing parallel no RT control reactions.

The expressions of these mRNAs were normalized using β-actin mRNA. The PCR amplification was performed on an ICycler PCR System (Bio-Rad). The mass of total RNA for RT, the number of cycles and thermal profile for PCR, and MgCl$_2$, primer and dNTP concentrations were selected experimentally (data not shown). RT-PCR products were resolved by electrophoresis in 2% agarose gels followed by ethidium bromide staining. Specific target amplification was confirmed by automatic sequencing (Macrogen, Seul, Korea).

Quantitative PCR (qPCR)

qPCR analysis was carried out using an ABI Prism 7500 detection system (Applied Biosystems) and SYBR green chemistry as described (Alamino et al. 2015). qPCR efficiency for each pair of primers was calculated using standard curves generated by serial dilutions of cDNA from DCs. All qPCR efficiencies ranged between 96% and 102% in different assays. mRNA levels are shown normalized against the housekeeping gene β-actin mRNA. We also used TATA box-binding protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize target mRNAs obtaining similar results (data not shown).

Preparation of total cell extracts

Cells were resuspended in RIPA buffer with protease inhibitors, disrupted by passages through a 25-G needle and incubated on ice followed by removal of DNA and debris by centrifugation. The supernatant was frozen at –80°C. Protein concentration was measured by the Bradford method (Bradford 1976).

Western blot analysis

Total cell lysates of DCs were used for immunodetection of MCT10 (55 kDa), LAT2 (58 kDa), D2 (31 kDa) and D3 (32 kDa) by Western blot analysis performed as described

Table 1 Mouse primers for conventional RT-PCR and RT-qPCR (SYBR GREEN) analysis.

Name	Primer	Name	Primer
Mct8 (NM_009197.2)	Forward: CCCATTGCATTTGAGCTGGTG Reverse: TTGATGCATCAGAGGGACGAAG	Lat2 (NM_016972.2)	Forward: GATAGTCCTTCGCTGGAAGAAGC Reverse: GGGTTTGTGTTGCCAGTAGACAC
Mct10 (NM_001114332.1)	Forward: CTAGTTGGTCCTCAGGATGCTTC Reverse: GGGATTAAACAGAGCACAACACC	Dio1 (NM_007860.3)	Forward: GTGGTGGACACAATGCAGAAC Reverse: GTGTCTAGGTGGAGTGCAAAGC
Oatp1c1 (NM_013797.5)	Forward: GTGGGGACAATGGGATTACC Reverse: CTTTCTGACACCTGCCCATC	Dio2 (NM_010050.2)	Forward: GCCACCTGACCACCTTTCA Reverse: CTTGGTTCCGGTGCTTCTTAA
Lat1 (NM_011404.3)	Forward: TCAGCTTCTTCAACTGGCTGTG Reverse: GCTTGTTCTTCCACCAGACACC	Dio3 (NM_172119.2)	Forward: AACTCTAGCAGTTCCGCATATGG Reverse: CCCACCAATTCAGTCACTTGTC

(Alamino *et al.* 2015). Abs against MCT10 (ab171649), LAT2 (ab75610), D2 (ab135711) and D3 (ab82041) (Abcam) were used. β-actin (Sigma) was used as loading control. To note, positive controls were selected in accordance to the data sheet provided by the supplier, but Western blots lacked a negative control. Signals were detected using the Odyssey Imaging System (LI-COR, Lincoln, NE, USA). Band intensities were evaluated using ImageJ software (NIH).

TH transport experiments

TH transport through DCs' membranes was evaluated as described (Visser *et al.* 2009, Loubiere *et al.* 2012, Zevenbergen *et al.* 2015). In brief, cells were washed with incubation medium (Dulbecco's PBS containing 0.1% D-glucose and 0.1% BSA). Uptake of THs was tested by incubation (3–90 min) of the cells at 37°C with 1 nM (100 × 10⁵ cpm) [^{125}I]T$_3$ or [^{125}I]T$_4$ in 1.5 mL incubation medium. Thereafter, cells were thoroughly washed with TH-free medium, lysed with 0.1 M NaOH and radioactivity was measured in a γ-counter. The uptake of [^{125}I]T$_3$ or [^{125}I]T$_4$ through TH transporters was inhibited by nonradioactive iodothyronines, bromsulfophthalein (BSP, MCT10 inhibitor) and/or leucine (Leu, LAT2 inhibitor) at concentrations previously reported (Visser *et al.* 2009, Loubiere *et al.* 2012). The Michaelis–Menten constant (K_m) and the half maximal inhibitory concentration (IC$_{50}$) were calculated from the GraphPad Prism (GraphPad Software 5.01). Dose–response inhibition curve was obtained by plotting the uptake percentage vs unlabeled THs concentration (Feng *et al.* 2015).

For measurement of TH efflux of TH transport at DC level, we followed the methods previously described (Visser *et al.* 2009). Cells were loaded for 30 min with incubation medium containing 1 nM [^{125}I]T$_3$ or [^{125}I]T$_4$, briefly washed with incubation medium, and subsequently incubated (0–60 min) with efflux medium (Dulbecco's PBS containing 0.1% D-glucose and 1% BSA). After incubation, cells were washed with incubation medium, lysed with 0.1 M NaOH and radioactivity was measured in a γ-counter. To allow evaluation of Na$^+$ dependence of the [^{125}I]T$_3$ or [^{125}I]T$_4$ uptake, cells were incubated in a buffer containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 1.8 mM CaCl$_2$, 20 mM HEPES and 0.1% BSA (pH 7.4) or in medium in which Na$^+$ was replaced with an equimolar concentration of choline (Visser *et al.* 2009).

Cell death assays

Cell death by necrosis and/or apoptosis of BSP and Leu-treated DCs was measured by flow cytometry using the PE–Annexin V-binding assay and 7-aminoactinomycin D (7-AAD; BD Biosciences), as we previously described (Alamino *et al.* 2015).

D2 and D3 activity assays

The activities of D2 and D3 were evaluated as previously described for other cellular systems (Wajner *et al.* 2011, Louzada *et al.* 2014), respectively. For D2 activity determination, DCs' samples (5 × 10⁶ cells/each) were homogenized in 150 mM sodium phosphate buffer containing 1 mM EDTA, 250 mM sucrose and 10 mM DTT (pH 6.9). Forty micrograms of protein of total homogenates from DCs were incubated in duplicate for 3 h at 37°C with [^{125}I]T$_4$ previously purified with Sephadex LH-20, 1 mM propylthiouracil (PTU, to inhibit any D1 activity) and 20 mM DTT in 100 mM potassium phosphate buffer (pH 6.9) containing 1 mM EDTA and 1 nM of T$_4$ in a final reaction volume of 300 μL. Specific D2 activity measurements were confirmed by the inhibition of deiodination reaction in the presence of T$_4$ in excess (100 nM). Blank incubations were carried out in the absence of protein. The reaction was stopped at 4°C in an ice bath with the addition of 200 μL FCS (Gibco) and 100 μL trichloroacetic acid (50%, v/v) followed by vigorous agitation. The samples were centrifuged at 10,000g for 3 min, and 360 μL of supernatant was collected for the measurement of ^{125}I liberated during the deiodination reaction, using a γ-counter. D2 activity was expressed as deiodinated T$_4$ in fmol/min/mg protein. Of note is that we used 3′-5′-[^{125}I]T$_4$ as substrate for D2 measurements, and thus, only the outer ring deiodination was detected. Regarding D3 activity measurement, cells were homogenized and sonicated with 10 mM Tris–HCl, 250 mM sucrose buffer (pH 7.5) and 10 mM DTT. The homogenates were incubated for 1 h with 200,000 cpm [^{125}I]T$_3$, 2 nM T$_3$, 20 mM DTT, and 1 mM PTU. The addition of 200 nM of T$_3$ completely abolished D3 activity in all samples (data not shown). The reaction was stopped by adding 200 μL ethanol 95%, 50 μL NaOH (0.04 M) and 5 mg PTU. Deiodination was determined based on the amount of [^{125}I]T$_2$ produced after reaction products separation by paper chromatography. Results were expressed as the fraction of T$_2$ counts minus the nonspecific deiodination (always <1.5%), obtained with the saturating concentration of T$_3$ (200 nM).

D3 activity was expressed as $[^{125}I]T_2$ in fmol produced/min/mg protein. The quantity of protein assayed was adjusted to ensure that <30% of the substrate was consumed.

Statistical analysis

The analysis of intergroup differences (three or more groups) was conducted by one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test. For the analysis of differences between two groups, the statistical significance was determined using the Student's t test for unpaired observations. P values less than 0.05 were considered statistically significant. All experiments were performed at least in triplicate.

Results

T_4 did not induce DC maturation and function, as T_3 does

We demonstrated relevant findings regarding T_3 action in the initiation of adaptive immunity (Mascanfroni et al. 2008, 2010, Montesinos et al. 2012, Alamino et al. 2015). However, T_4 is the main TH secreted by the thyroid and the major TH in circulation, fourfold higher considering free hormone levels (Williams & Bassett 2011). Therefore, the effect of T_4 on sensitive DC maturation/function parameters was assayed. For this purpose, murine bone marrow-derived immature DCs (iDCs) were treated with T_4 (from sub- to supra-physiological levels, as found in mice circulation), and IL-12 production and secretion were measured following the experimental protocol carried out with T_3 (Mascanfroni et al. 2008). As shown in Fig. 1, T_4 at 10^{-8}–10^{-6}M for 18h did not induce any significant modification in IL-12 production by DCs (Fig. 1A) or IL-12 secreted to the medium (Fig. 1B), as T_3 does. Time course studies (18–36h) neither revealed any significant change (Fig. 1C) in IL-12 released. These results show that TH endowment of DCs with enhanced ability to simulate T-cell responses is depending on T_3.

MCT10 and LAT2 transporters are expressed in DCs and transport THs, mainly T_3

To further delve into TH action at DC level, the study of TH transport across the DC cell membrane became mandatory. At first, we examined the expression of MCT8,
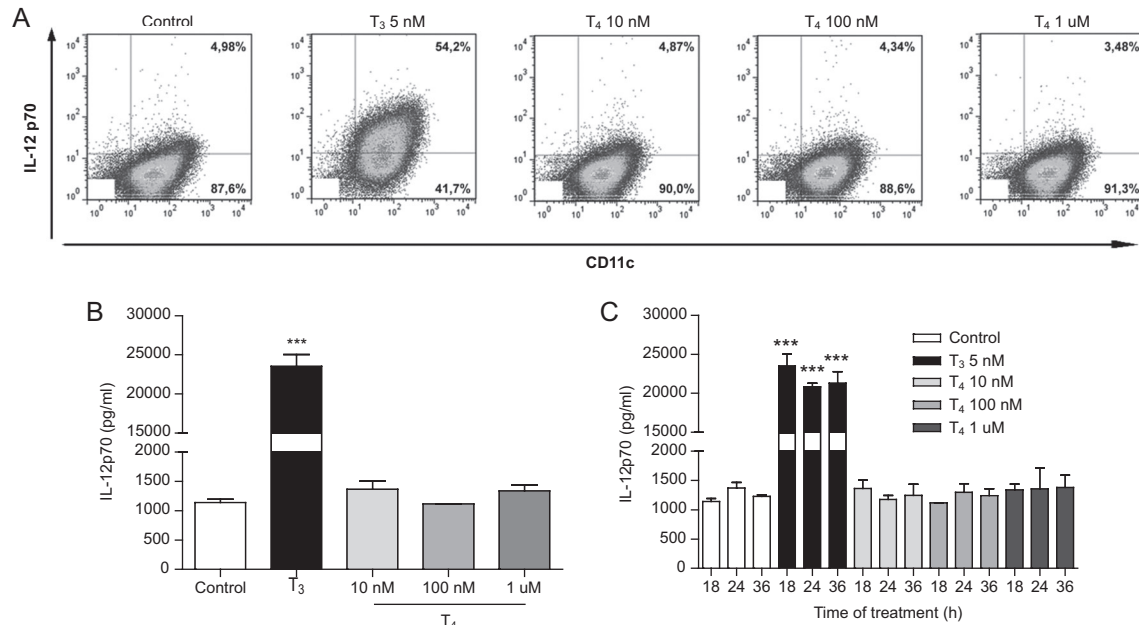

Figure 1

Effects of T_4 on IL-12 production and secretion by DCs. (A, B) iDCs were incubated with medium alone (Control), T_3 (5nM) or T_4 (10nM, 100nM or 1M) for 18h. (A) Intracellular IL-12 was analyzed by flow cytometric analysis of DCs using PE-conjugated anti-IL-12. Representative dot plots of 3 independent experiments conducted in triplicate are shown. (B) IL-12 production was determined in culture supernatants by ELISA. Data are expressed as mean±S.D. of a representative experiment of 3 with similar results performed in triplicate. ***$P<0.001$ vs iDC (Control). (C) Time course study of the effect of T_4 on IL-12 secretion. iDCs were incubated with T_3 (5nM) or T_4 (10nM, 100nM and 1M) for 18, 24 and 36h and IL-12 was measured in the culture media by ELISA. Data are expressed as mean±S.D. of a representative experiment of 3 with similar results performed in triplicate. ***$P<0.001$ vs iDC (Control) at each time of T_3 treatment, respectively.

MCT10, OATP1C1, LAT1 and LAT2 in iDCs, at mRNA and protein levels, by conventional RT-PCR and Western blot, respectively. Figure 2A shows that iDCs express mRNAs for *Mct10* and *Lat2*, but not for *Mct8*, *Oatp1c1* or *Lat1*. These results are in agreement with MCT10 and LAT2 protein expression (Fig. 2B) at comparable levels, although at lesser extent than their positive controls (K562 and HeLa cells). Altogether, these findings highly suggest that MCT10 and/or LAT2 may be involved in TH transport into DCs.

We next attempted to further characterize the process of TH transport at DC level. We assessed $[^{125}I]T_3$ and $[^{125}I]T_4$ (1 nM) uptake by iDCs, as previously reported (Visser *et al.* 2009). Time course analysis demonstrated that both THs were transported by these cells, although cellular uptake of $[^{125}I]T_3$ was significantly higher than that of $[^{125}I]T_4$ at all times evaluated, about 5.7 times at 90 min (Fig. 3A) and revealing that at equal concentrations, the main TH that gets into DCs is T_3. Of note is that the rate of $[^{125}I]T_3$ uptake (%) increased linearly for 30 min and decreased thereafter, in a similar fashion to other TH-responsive cells (Visser *et al.* 2009). Afterwards, we evaluated the effects of increasing concentrations of unlabeled iodothyronines on $[^{125}I]T_3$ uptake. As depicted in Fig. 3B, a dose-dependent reduction of $[^{125}I]T_3$ uptake was observed after the addition of increasing concentrations of unlabeled iodothyronines, T_3 or T_4. As shown, there are two uptake processes,

in agreement with other cellular systems (Visser *et al.* 2009, Loubiere *et al.* 2012). In this regard, one of them is not saturated for T_3 or T_4 concentrations up to 10 μM, whereas the saturable $[^{125}I]T_3$ uptake process shows a K_m value for T_3 inhibition of 0.76 μM and the IC_{50} value for T_4 inhibition of T_3 uptake of 7.78 μM. Considering that $[^{125}I]T_4$ uptake was much lower than $[^{125}I]T_3$ uptake (Fig. 3B), the proper determination of kinetic parameters for $[^{125}I]T_4$ uptake was not possible. As MCT10 and LAT2 are sodium-independent transporters (Halestrap & Wilson 2012, Kinne *et al.* 2015), we investigate the dependence of T_3 uptake on sodium. Figure 3C shows that replacement of sodium by choline did not induce any significant reduction of $[^{125}I]T_3$ uptake, indicating that T_3 transport system does not have any sodium dependence, providing further evidence for MCT10 and/or LAT2 involvement in T_3 transport at DC level. Besides, the efflux of $[^{125}I]T_3$ from DCs has a significantly lower velocity than $[^{125}I]T_4$ (Fig. 3D) as about 63% of cellular $[^{125}I]T_3$ remains inside DCs at 60 min after its uptake vs 32% of $[^{125}I]T_4$.

To further characterize the T_3 uptake system at DC level, it was measured in the presence of MCT10 and LAT2 inhibitors: BSP and Leu, respectively (Loubiere *et al.* 2012) (Fig. 4A). As shown, BSP significantly reduced 54% of total $[^{125}I]T_3$ uptake, whereas Leu decreased it just 15%, without significant difference from control cells. When BSP and Leu were added together, $[^{125}I]T_3$ uptake
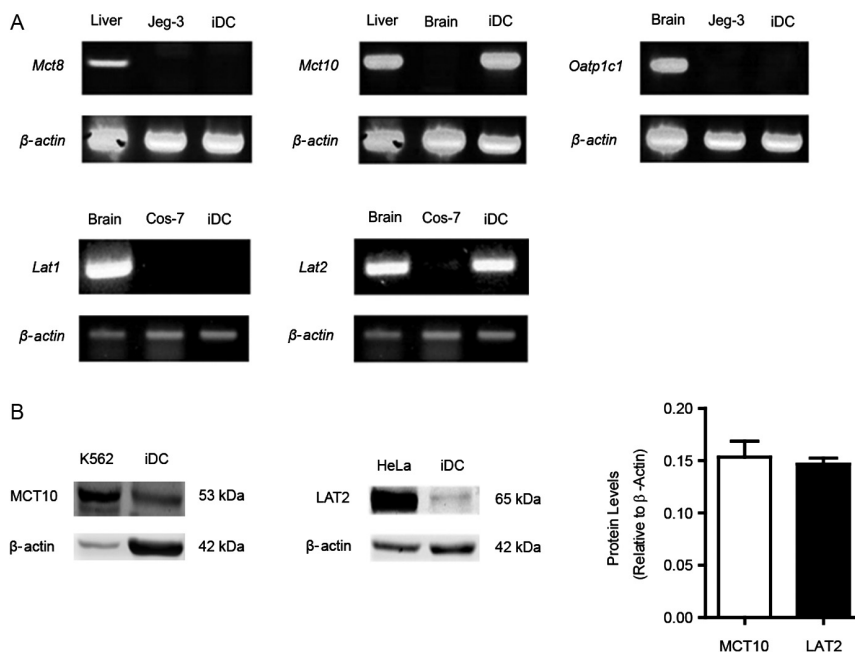
Figure 2

Expression of TH transporters in DCs. (A) Detection of mRNAs for *Mct8* (left upper panel), *Mct10* (medium upper panel), *Oatp1c1* (right upper panel), *Lat1* (left lower panel) and *Lat2* (medium lower panel) by conventional RT-PCR analysis of total RNA from iDCs. Total RNA from mouse liver, mouse brain, COS-7 and JEG-3 cells were run in parallel as positive (first line) or negative (second line) controls, respectively. β-actin mRNA was run as a loading control. RT-PCR products were resolved by electrophoresis in 2% agarose gels followed by ethidium bromide staining. (B) Representative Western blot analysis of total cell lysates from iDCs for MCT10 (left panel) and LAT2 (medium panel). Lysates from K562 and HeLa cells were run in parallel as positive controls of MCT10 and LAT2, respectively. Samples were separated by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and blotted with anti-MCT10 or anti-LAT2 Abs. Equal loading was checked using an anti-β-actin mAb. Representative blots of 3 independent experiments, conducted in triplicate and with similar results are shown. Right panel shows quantitative analysis of Western blots. Data are expressed as mean ± s.d. of a representative experiment from 3 with similar results and performed in triplicate.
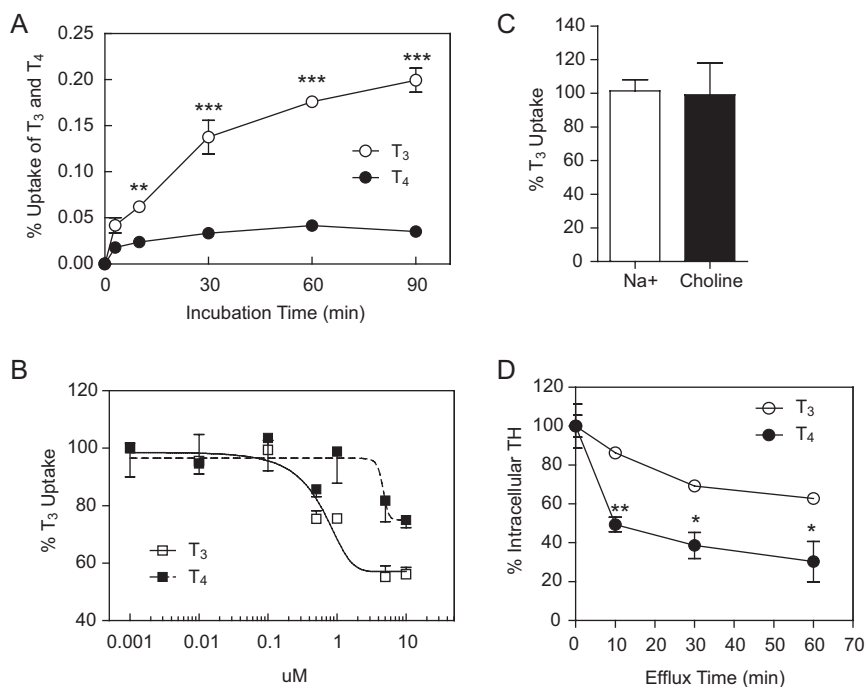
Figure 3

Characterization of TH transport at DC level. (A) Time course of 1 nM [^{125}I]T$_3$ (O) and [^{125}I]T$_4$ (●) uptake in mice bone marrow-derived iDCs. THs uptake was measured in D-PBS containing 0.1% glucose and 0.1% BSA. **$P < 0.01$, ***$P < 0.001$ vs [^{125}I]T$_4$ at the same time of incubation. (B) Effects of increasing concentrations (1 nM–10 μM) of unlabeled iodothyronines (□ T$_3$, ■ T$_4$) on [^{125}I]T$_3$ uptake in iDCs. (C) Na$^+$ dependence of [^{125}I]T$_3$ uptake in DCs: Cells were incubated with 1 nM [^{125}I]T$_3$ for 30 min in sodium chloride medium (white bar) or sodium-free choline medium (black bar). Results are expressed as mean ± S.D. of the percentage of [^{125}I]T$_3$ uptake from cells incubated with Na$^+$ containing media. (D) TH efflux from DCs. Cells were incubated for 30 min with [^{125}I]T$_3$. Afterward, media was removed, cells were thoroughly washed and [^{125}I]T$_3$ measured at 0, 30 and 60 min thereafter. *$P < 0.05$, **$P < 0.01$ vs [^{125}I]T$_3$. Results are expressed as mean ± S.D. of [^{125}I]T$_3$ uptake at 0 min and are from a representative experiment from a total of 3 with similar results performed in triplicate.

decreased 45%; however, the difference between BSP and BSP+Leu treatments failed to reach statistical significance. Altogether, these findings are in agreement with T$_3$ inhibition of the saturable process (Fig. 3B), highly suggesting that MCT10 is the main TH transporter. To evaluate the biological significance of MCT10 and LAT2 for T$_3$ action in DCs, IL-12 secretion induced by T$_3$ (Mascanfroni *et al.* 2008) was measured after concomitant treatment for 18 h (or not) with both inhibitors. As shown in Fig. 4B, the addition of Leu in the presence of T$_3$ did not

induce any significant change on IL-12 secretion by DCs. However, BSP reduced the effect of T$_3$ by 46%, whereas the presence of both inhibitors together did not diminish IL-12 levels below BSP alone. As treatment with T$_3$+BSP did not abolish the effect of T$_3$ on IL-12 secretion by DCs, the amount of T$_3$ that enters DCs by the non-saturable mechanism or by other unknown TH transporters is enough to induce, although to a lesser extent, the ability of DCs to secrete IL-12. To note, neither BSP nor Leu (at the concentrations assayed) induced any significant
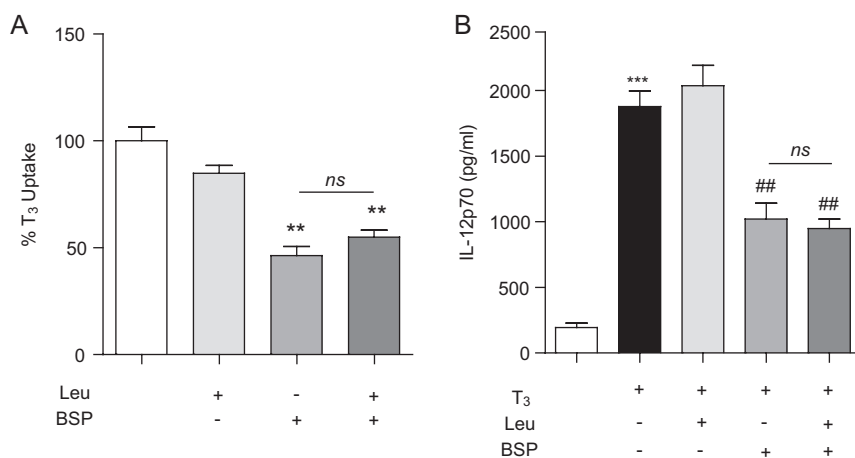

Figure 4

Effect of MCT10 and LAT2 inhibitors on T$_3$ uptake (A) and IL-12 secretion (B) by DCs. (A) Effect of 100 μM unlabeled competitors: BSP, Leu or both on [^{125}I]T$_3$ uptake. DCs were incubated for 30 min with [^{125}I]T$_3$ in the absence or the presence of competitors, and net uptake was measured thereafter. Results are expressed as mean ± S.D. from the percentage of control uptake (1 nM [^{125}I]T$_3$) and are from a representative experiment from a total of 3 with similar results conducted in triplicate. **$P < 0.01$ vs control DCs. (B) Effect of 100 μM competitors: BSP, Leu or both on T$_3$-induced IL-12 secretion by DCs. DCs were incubated for 18 h with T$_3$ with or without mentioned inhibitors. IL-12 production was determined in culture supernatants by ELISA. Results are expressed as mean ± S.D. and are from a representative experiment from a total of 3 with similar results conducted in triplicate. ***$P < 0.001$ vs Control DCs, ##$P < 0.01$ vs T$_3$ alone, ns: no significant.

effect on DC viability over the experimental time (data not shown), measured by the cell death assay. These results highlight the involvement of MCT10 on T_3 action at DC level and also disregard LAT2 participation.

Types 2 and 3 iodothyronine deiodinases are expressed in DCs and metabolize THs

Considering the pivotal role of TH metabolism in the action of THs in target cells (Williams & Bassett 2011), we explored this event in DCs. We first evaluated the expression of TH deiodinase mRNAs (*Dio1*, *Dio2* and *Dio3*) in iDCs, by conventional RT-PCR. Figure 5A shows that iDCs express *Dio2* and *Dio3*, but not *Dio1*. In turn, these cells also exhibit D2 and D3 protein expression, measured by Western blot (Fig. 5B) that revealed the monomer 31 kDa for D2 and the 65 kDa homodimer native state for D3 (Sagar *et al*. 2008). Monomeric D3 could not be obtained, even after urea (6 M) treatment of protein lysates. Of note is that D3 protein levels are significantly higher than those of D2 (6.5 fold, Fig. 5B).

To evaluate DC's ability to control intracellular levels of THs, D2 and D3 activities were assessed in iDCs. The activities of D2 and D3 vs protein mass were determined, and results indicated that the increase of D2 activity is linear between 20 and 60 μg, whereas it is from 80 to 120 μg for D3 (data not shown). Therefore, 40 μg (for D2)

and 100 μg (for D3) of total protein extracts were used thereafter. As depicted in Fig. 5C, D2 and D3 activities were revealed in iDCs, although $[^{125}I]T_3$ (black bar, D3 activity) was deiodinated at a higher extent than $[^{125}I]T_4$ (white bar, D2 activity). The higher D3 than D2 activity (6.6-fold) is quite similar to D3 vs D2 protein expression in iDCs. Altogether, these results suggest that D3 may be preponderant over D2 at least in control (immature) conditions, revealing a major role of iodothyronine inactivation in iDCs.

Regulation of TH transporters and iodothyronine deiodinases by T_3

As T_3 exhibits profound effects at DC level, inducing phenotypic and functional maturation of iDCs and regulating thereafter adaptive immunity (Mascanfroni *et al*. 2008, 2010, Montesinos *et al*. 2012, Alamino *et al*. 2015), the T_3 effect (5 nM for 18 h) on TH transporters and iodothyronine deiodinases 2 and 3 were evaluated.

As depicted in Fig. 6A, RT-qPCR analysis shows that T_3 significantly increased *Mct10* and *Lat2* mRNAs. These results are in accordance with protein levels of both TH transporters (Western blot, Fig. 6B). In turn, $[^{125}I]T_3$ and $[^{125}]T_4$ uptake by control iDCs and T_3-treated cells revealed that although T_3 uptake was not modified by T_3 treatment
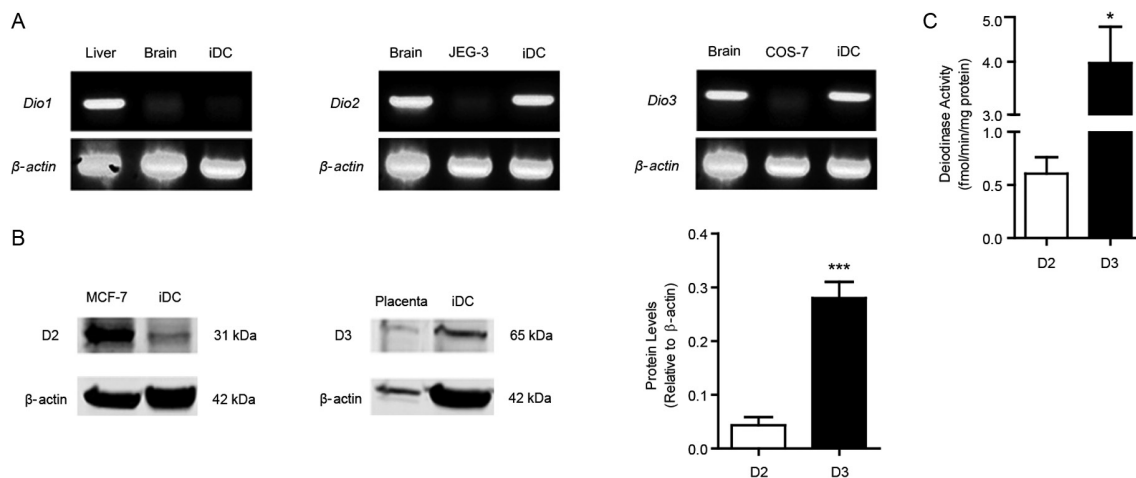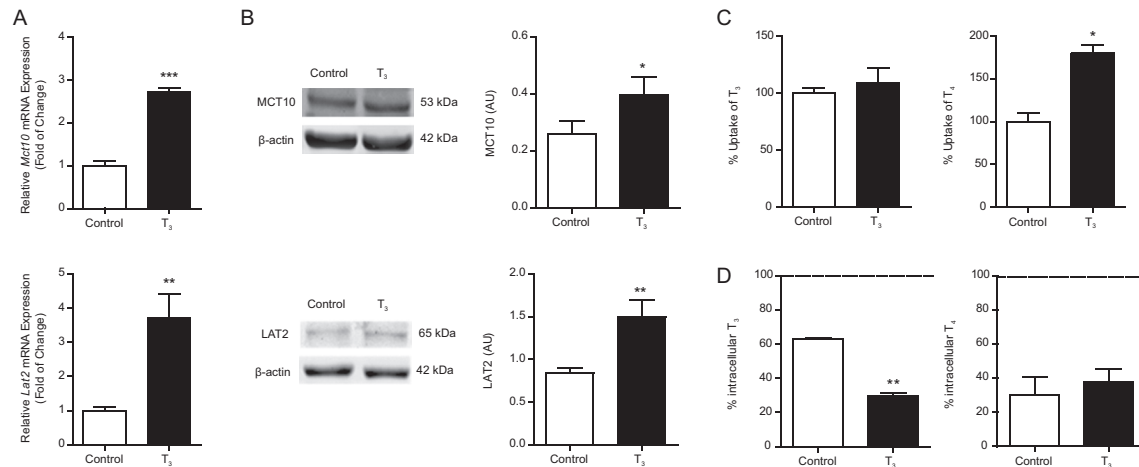

Figure 5
Expression and activity of iodothyronine deiodinases in iDCs. (A) Detection of mRNA for type 1, 2 and 3 iodothyronine deiodinase mRNAs (*Dio1*, *Dio2* and *Dio3*) in iDCs by conventional RT-PCR. Total RNA from mouse liver, mouse brain, JEG-3 and COS-7 cells were run in parallel as positive (first line) or negative (second line) controls. β-actin mRNA is shown as a loading control. RT-PCR products were resolved by electrophoresis in 2% agarose gels followed by ethidium bromide staining. (B) Representative Western blot analysis for D2 (left panel) and D3 (medium panel) in total cell lysates from iDCs. Lysates from MCF-7 cells (positive control for D2) and rat placenta (positive control for D3) were run in parallel. Samples were separated by 10% SDS-PAGE, transferred onto nitrocellulose membranes and blotted with anti-D2 or anti-D3 Abs. Equal loading was checked using an anti-β-actin mAb. Third panel shows densitometric analysis of Western blots. ***$P<0.001$ vs D2 protein expression. (C) D2 and D3 activities in iDCs. Results are indicated as fmol T_4 (for D2) or T_2 (for D3)/min/mg protein. Data are expressed as mean ± s.d. and are from a representative of 3 experiments conducted in triplicate. *$P<0.05$ vs D2.

Figure 6

Effect of T_3 treatment to iDCs on MCT10 and LAT2 levels. MCT10 and LAT2 at mRNA and protein levels, and T_3 or T_4 uptake was evaluated in Control (iDC) or iDCs treated with 5 nM T_3 for 18 h (T_3). (A) RT-qPCR analysis of *Mct10* (upper panel) and *Lat2* (lower panel) mRNAs. The levels of *Mct10* and *Lat2* mRNAs were quantified relative to those of β-actin. Results are indicated as fold of change relative to the mRNA levels of Control iDC. **$P < 0.01$; ***$P < 0.001$ vs Control. (B) Western blot analysis of MCT10 and LAT2 protein levels. Left panels show representative blots for MCT10 (upper panel) and LAT2 (lower panel). Equal loading was checked with β-actin. Right panels exhibit the densitometric analysis of Western blots. Data are expressed as mean ± S.D. (arbitrary units, AU) and are from a representative of 3 experiments with similar results and conducted in triplicate. *$P < 0.05$; **$P < 0.01$, vs Control. (C) Uptake of [125I]T_3 (left panel) and [125I]T_4 (right panel). *$P < 0.05$ vs Control. (D) Efflux of [125I]T_3 (left panel) and [125I]T_4 (right panel) from iDCs and T_3-treated DCs. Data are expressed as mean ± S.D. and are from a representative of 3 experiments performed in triplicate. **$P < 0.01$ vs Control.

of iDC, T_4 uptake was significantly increased 2.1-fold (Fig. 6C). On the contrary, T_3 efflux was significantly increased in T_3-treated DCs when compared to control iDCs (Fig. 6D), whereas T_4 efflux was not modified (Fig. 6D).
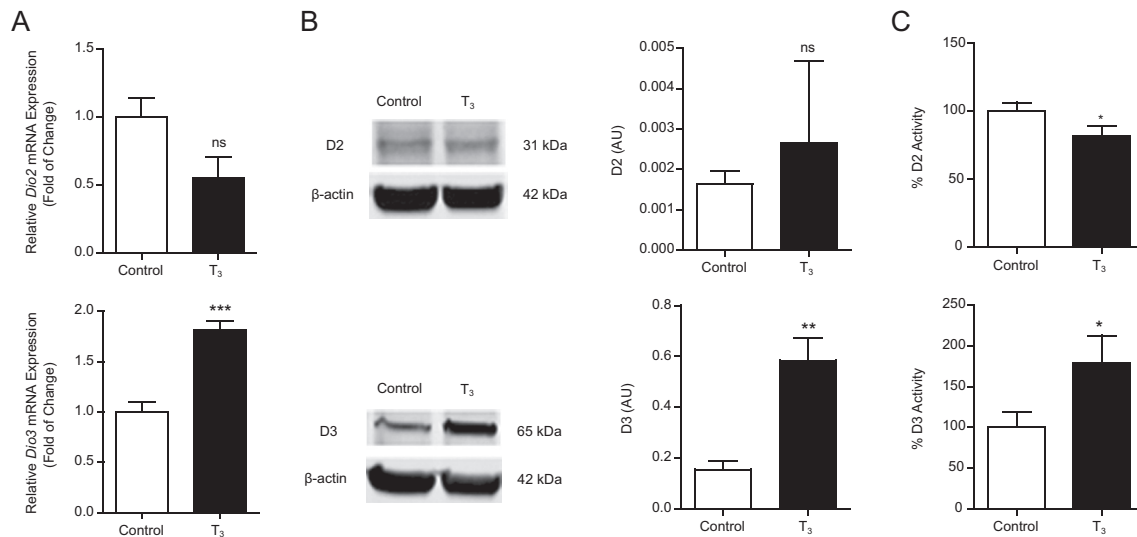
Regarding T_3 effect on iodothyronine deiodinase expression in DCs, T_3 significantly augmented *Dio3* mRNA expression (1.81 fold increase vs control iDCs, Fig. 7A) and D3 protein levels (3.74 fold increase vs control iDCs, Fig. 7B). In addition, D3 activity was increased by T_3 treatment (1.78 fold increase vs control iDCs, Fig. 7C). Conversely, *Dio2* mRNA levels tended to decrease in T_3-treated DCs, but this failed to reach statistical significance (Fig. 7A). In turn, D2 protein levels were not modified by T_3 treatment (Fig. 7B) although D2 activity registered a slight but significant decrease (18%, Fig. 7C, upper panel).

Discussion

During the last decade, our findings underscore a novel unrecognized target that regulates DC maturation and function, T_3, with outstanding effects at the initiation of adaptive immunity (Mascanfroni *et al.* 2008, 2010, Montesinos *et al.* 2012, Alamino *et al.* 2015). At present, the players involved in TH actions are well characterized. However, the interaction among them remains to be

fully elucidated in a tissue-specific fashion (Brent 2012). As the action of THs may represent an attractive target for rational manipulation of the immunogenicity of DCs, either alone or in combination with other cancer immunotherapeutic strategies (Alamino *et al.* 2016), the effect of the main circulating TH (T_4), as well as the mechanisms of TH transport and metabolism at DC levels, were disclosed in this work. Noteworthy, both mechanisms were scarcely and only partially studied in few cells from the immune system up to date (Boelen *et al.* 2008, 2009b, Stevenson *et al.* 2014), and therefore, this is the first systematic study in the field, conducted at DC level.

In many cellular systems, T_4 acts mainly as a prohormone through intracellular T_3 conversion to provide the major part of circulating T_3 levels and to begin TH action inside target cells, although rapid nongenomic effects initiated mainly by T_4 at the plasma membrane were also reported (Davis *et al.* 2016). The source of cellular T_3, from plasma or from local T_4 deiodination by D2, is extremely variable in different tissues, e.g. 80% is derived from plasma in cerebral cortex, whereas less than 20% is from this fraction in kidneys (Dayan & Panicker 2009). Our results showing that different from T_3 (Mascanfroni *et al.* 2008), treatment of DCs with T_4 has no effect on IL-12 production and secretion, gave us the first clue that DC response to

Figure 7

Effect of T_3 treatment to iDCs on iodothyronine deiodinases 2 and 3. *Dio* 2 and 3 mRNAs, and D2 and D3 protein levels as well as D2 and D3 enzyme activities were evaluated in Control (iDC) or T_3-treated (T_3) iDCs. (A) RT-qPCR analysis of *Dio2* (upper panel) and *Dio3* (lower panel) mRNAs. The levels of *Dio2* and *Dio3* mRNAs were quantified relative to those of β-actin. Results are indicated as fold of change relative to the mRNA levels of Control iDC. ***$P < 0.001$, vs Control. (B) Western blot analysis of D2 and D3. Left panels show representative Western blots for D2 (upper panel) and D3 (lower panel). Equal loading was checked with β-actin. Right panels exhibit the densitometric analysis of Western blots. Data are expressed as mean±s.d. (arbitrary units, AU) and are from a representative of 3 experiments with similar results and conducted in triplicate. **$P < 0.01$, vs Control. (C) D2 (upper panel) and D3 (lower panel) enzyme activities. Data are expressed as mean±s.d. and are from a representative of 3 experiments performed in triplicate. *$P < 0.05$ vs Control.

TH may be due, at least in its major part, directly by T_3 action. In some tissues, like the central nervous system, the T_3 derived from T_4 deiodination also acts in other cells, different from those where it was produced (St Germain *et al.* 2009). Therefore, although T_4 is the major circulating TH, T_3 may also be supplied to DCs from other neighbor cells in their sites of maturation, e.g. lymph nodes and spleen. Considering the crucial reported events induced by T_3 on DC characteristics and function (Mascanfroni *et al.* 2008, Alamino *et al.* 2015), this postulate seems rational to restrain unspecific activation of DCs in circulation. Moreover, as the biological activity of THs is dependent on the intracellular T_3 concentration, which is importantly controlled by TH transport across DCs' membranes and TH metabolism inside target cells (Bernal *et al.* 2015), further results from this work shed light on both events at DC level, crucial for the fate of the immune system exposed to pathogens or damage signals.

Results show that both MCT10 and LAT2 TH transporters are expressed at mRNA and protein levels in DCs. It was reported that MCT10 is as active as MCT8 for TH transport (Friesema *et al.* 2008), the main TH transporter and most studied due to its brain expression leading to the severe Allan–Herndon–Dudley syndrome when its gene is mutated. Remarkably, although scarce

information about TH transporter expression in immune cells is found, the macrophage cell line RAW264.7 also expresses *Mct10* (at mRNA level) but not *Mct8* or *Oatp1c1* (Kwakkel *et al.* 2014), in consonance with our results. Noteworthy, macrophages are phylogenetically and functionally related to DCs and it was reported that mice macrophages also exert T_3-dependent effects (Perrotta *et al.* 2013), although TH transport has not been evaluated in these cells yet.

Further analysis of TH transport mechanism revealed that DCs are more effective in transporting T_3 than T_4, in agreement with MCT10 and LAT2 expression and regarding their iodothyronine transport preference (Friesema *et al.* 2008, Loubiere *et al.* 2012, Schweizer *et al.* 2014). In accordance, our results show that T_3 uptake in DCs is sodium independent (Halestrap & Wilson 2012, Kinne *et al.* 2015).

Regarding the kinetic characteristics of TH transport, we registered a saturable and a non-saturable component, in tight agreement to TH transport in other cells (Visser *et al.* 2009, Loubiere *et al.* 2012). It was suggested that the latter may reflect iodothyronine partitioning in the cell membrane and/or an unknown transporter system with K_m values in the millimolar range (Visser *et al.* 2009). When unlabeled T_3 is added, the saturable process shows a dose-dependent reduction in [^{125}I]T_3 uptake

with an apparent K_m 10 times below the concentration of T_4 needed to inhibit 50% of this uptake (0.76 µM vs 7.78 µM). These kinetic results provide more evidence to the preferable transport of T_3 over T_4 at DC level, and give an additional support to the undetectable effect of T_4 on DC function.

Results of the study of T_3 transport in DCs in the presence of selective MCT10 and LAT2 inhibitors strengthen the main role of MCT10 in T_3 transport in DCs. In agreement, it was reported that LATs exhibit lower affinity than MCTs to transport THs (Friesema et al. 2005) and a recent report revealed that LAT2 exhibits favored affinity for 3,3′-diiodothyronine (T_2), less for T_3 and no affinity for T_4 (Kinne et al. 2015), reinforcing the evidence that LAT2 is not compelling for T_3 crossing through DC's membrane. Noteworthy, it was reported that MCT10 transports T_3 and T_4 in and out of cells (Friesema et al. 2008), but it was suggested that it plays a prominent role in T_3 efflux (Muller et al. 2014). Considering MCT10 preference for T_3 transport, the higher efflux of T_4 than T_3 out of DCs may be due to other modulators of TH mechanism of action inside the cell, like the cytoplasmic T_3-binding protein or µ-crystallin: CRYM, which binds T_3 with higher affinity than T_4 and is a regulator of intracellular T_3 bioavailability (Suzuki et al. 2007). Remarkably, the marked reduction of IL-12 secretion from DCs exposed to T_3 + BSP over T_3 alone reinforces and gives functional support for MCT10's prominent role on T_3 transport in DCs.

Although both Mct10 and Lat2 mRNA and protein levels were increased by T_3 treatment of DCs, T_3 efflux velocity (but not T_3 uptake) was augmented. These findings may respond to the preferable role of MCT10 on T_3 efflux (Muller et al. 2014). Conversely, T_4 uptake was increased by T_3-treated DCs. Although under this circumstance, T_4 entry to DCs may be favored as a compensatory mechanism to counteract the fall in T_3 intracellular level, the amount of T_4 uptake is significantly lesser than that of T_3. Overall, these findings would be involved in T_3 clearance from DCs and sustain a homeostatic mechanism to restrain TH action after DC activation.

Although TH cellular concentrations are variable depending on the specific metabolic state, this situation does not impact at plasma view point as TH serum levels hardly fluctuate. Therefore, by a particular modulation of deiodinase expression and activity at cellular level, TH action is finely controlled (Bianco 2013). Although this mechanism should also operate at TH-responsive immune-type cells, just the expressions of deiodinases were reported in some cell types. These include D3 protein expression in granulocytes and macrophages from a rat model of experimental allergic encephalomyelitis (Boelen et al. 2009a), D3 immunoreactivity in inflammatory cells surrounding a local abscess induced by turpentine injection to mice (Boelen et al. 2008), Dio2 mRNA expression at baseline levels in the macrophage cell line RAW264.7 with Dio1 and Dio3 mRNAs under the limit of detection (Kwakkel et al. 2014), and Dio2 and Dio3 mRNA expression in hamster leukocytes (Stevenson et al. 2014). However, as far as we know, the activities of deiodinases were not reported in any immune-type cell. As for TH transport, our results provide the first report of TH metabolism in murine DCs, covering the whole metabolic process from the expressions and the activities of iodothyronine deiodinases, to the regulation by T_3.

Our results showing iodothyronine deiodinase expression in DCs for types 2 and 3 but not for type 1 is not surprising as it resembles the profile of many TH-responsive cells since both D2 and D3 are mainly involved in the control of TH action at cellular level (Bianco 2013). Conversely, D1, a high Km enzyme (10^{-7}–10^{-6} M), mainly contributes to circulating T_3 levels and acts as a scavenging enzyme (Williams & Bassett 2011, Maia et al. 2011, Darras et al. 2015). Moreover, as D1 is anchored at the plasma membrane and exhibits low affinity for T_4, T_3 generated at this level diffuses rapidly from the cell to the plasma without significant modifications of intracellular levels of T_3 (Gereben et al. 2015). Noteworthy, D3 protein levels and enzyme activity are greater than D2 in DCs. Considering both enzymes share the same low Km in the nM range (Williams & Bassett 2011) but D3 has a longer half-life than D2 (Bianco & Kim 2006), a higher role of T_3 inactivation by D3 than T_3 intracellular generation by D2 activity on T_4 is suggested. These results seem expectable due to the scarce T_4 uptake by the cell and point out another step of cellular control of the active TH (T_3) in DCs, favoring its inactivation. As D3 also catalyzes T_4 inactivation rendering the inactive iodothyronine metabolite: rT_3, the little amount of T_4 entering DCs may be sensitive to inactivation. This mechanism together with the little T_4 uptake by DCs may collaborate to render the lack of T_4 effect at DC level. These results are of relevance since these mechanisms may be involved in the control of T_3 action at DCs to avoid the perpetuation of the proinflammatory response induced through the Th1 profile and cytotoxic T-cell activity triggered by T_3 (Mascanfroni et al. 2008, Alamino et al. 2015). Besides, taking together the results showing low T_4 uptake by DCs and reduced TH activation by D2 inside the cell, suggest that T_3 may be supplied to DCs by the extracellular medium and/or by D2 activity

on T_4 at neighboring cells in their activation sites (St Germain *et al.* 2009). The disclosure of this issue exceeds the objective of this work, but since DCs are expected to be in an immature state in circulation, it may be hypothesized that this second source of T_3 is of enough magnitude to modulate DC functioning and plays an important role to control the precise place of DC maturation.

Results showing that the expected effect of T_3 on the increased expression and activity of D3 was more evident than the reduction of D2 in DCs, indicate that the cellular activity of T_3 must be finely tuned in these cells. These results are in agreement with many authors (Bianco *et al.* 2002, Darras *et al.* 2015) that reported that fluctuations in T_4 and T_3 concentrations lead to homeostatic, reciprocal changes in the activity of D2 and D3. In fact, as T_3 concentrations increase, expression of *Dio*3 is upregulated, increasing T_3 clearance, whereas the expression of *Dio*2 is just modestly downregulated, decreasing T_3 production.

Worthy to be discussed, T_3 regulation of TH transporter and deiodinase expressions and activities may be the result of its known regulatory action on TH economy in target cells, depending on the physiological or pathological condition (Bianco 2013). However, an action of T_3 due to its role on DCs' maturation and function is also expected. Therefore, the results obtained in this work may be the result of both mechanisms. As different stimuli exert specific actions on DCs' characteristics and functioning, as well as particular effects on the ability of these cells to develop adaptive responses (Walsh & Mills 2013, Kudo *et al.* 2013), the disclosure of effects of many other DCs' stimulators on TH transporter and deiodinase expressions and activities, exceeds the goal of this manuscript and is being addressed in ongoing research.

Previously, we reported that T_3 increased TRβ1 expression in DCs through NFκB-positive action on the transcription of its gene (Mascanfroni *et al.* 2010) and that TRβ1 is essential for T_3 action *in vitro* (Mascanfroni *et al.* 2010) and *in vivo* (Alamino *et al.* 2015). Altogether, T_3 action on TH transport (uptake–efflux), iodothyronine deiodinases and TRβ1, may act coordinately to regulate TH action within the cell in a balanced fashion and depending on the functional requirements of DCs during different physiopathological conditions.

In conclusion, our results underscore in more detail the action of THs at DC level, revealing the pivotal role of T_3 over T_4 and disclosing the mechanisms of TH transport and iodothyronine deiodination in these cells. They also broaden the knowledge of the effect of THs at the initiation of the immune response and their role directing adaptive immunity through T_3 actions on DCs, with profound implications in immunopathology, including cancer and autoimmune manifestations of the thyroid gland at the crossroads of the immune and endocrine systems. Noteworthy, these results are relevant regarding the recorded effects of T_3 on DC functioning as the knowledge of TH transport and metabolism in DCs provides valuable tools for manipulating the immunogenic potential of DCs to positively regulate the development of protective immunity or negatively control the generation of autoimmune thyroid inflammation.

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