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 (T₃)-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 (T₄) 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 T₄ did not reproduce those registered T₃-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 T₃ with a favored involvement of MCT10 as its inhibition almost prevented T₃ saturable uptake mechanism and reduced T₃-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, T₃ increased MCT10 and LAT2 expression and T₃ efflux from DCs but not T₃ 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 (T₄). However, it provides a low percentage of serum levels of the most physiologically active TH: 3,5,3′-triiodothyronine (T₃), the major part of which is derived from peripheral 5′ deiodination of T₄ (Williams & Bassett 2011).

Cellular activity of THs is classified as genomic (nuclear) and nongenomic (initiated either in the...
cytoplasm or at the plasma membrane). However, the main mechanism of TH action requires the participation of T3 and its nuclear receptors (TRs): TRα1, TRβ1, TRβ2, and TRβ3, whereas other non-T3-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 T3 uptake than T4 (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 T4 to T3, whereas D3 deiodinates the inner ring iodides, thus initiating the conversion of T3 and T4 in inactive metabolites. Therefore, D2 is considered an ‘activating’ enzyme, responsible for the peripheral production of 50–80% of the total body pool of T3. Conversely, D3 restrains T3 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 T3 contributes to the maturation of the main APC: dendritic cells (DCs). A Th1-lymphocyte T adaptive response induced by physiologic levels of T3 was also revealed (Mascarenfoni et al. 2008). Mechanistically, these effects involved the activation of Akt and NF-kB pathways (Mascarenfoni et al. 2010) and were counteracted by glucocorticoids (Montesinos et al. 2012). Moreover, we recently revealed that T3 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 T4 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**

[125I]T3 and [125I]T4 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 (Mascarenfoni 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 T3 (5nM) for 18h (that induce DC maturation: mDCs) or with T4 at times and concentrations indicated. Parallel cultures were maintained without stimuli as controls. To rule out endotoxin contamination of T3 and T4 preparations, we checked the endotoxin content that raised levels lower than 0.03IU/mL (limit of detection) by the Limulus amebocyte lysate assay.
Flow cytometric analysis

Intracellular IL-12 and CD11c 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.

Cytokine determination

IL-12p70 detection was performed in cell culture supernatants using standard capture enzyme-linked immunosorbent assays (ELISA), as reported (Alamino 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 (Dio1, Dio2 and Dio3) mRNAs were from Sigma (Buenos Aires, Argentina). 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 MgCl2, 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...
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, Loubierre 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 [(125)I]T3 or [(125)I]T4 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]T3 or [(125)I]T4 through TH transporters was inhibited by nonradioactive iodothyronines, bromsulfophthalain (BSP, MCT10 inhibitor) and/or leucine (Leu, LAT2 inhibitor) at concentrations previously reported (Visser et al. 2009, Loubierre et al. 2012). The Michaelis– Menten constant (Km) and the half maximal inhibitory concentration (IC50) 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]T3 or [(125)I]T4 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]T3 or [(125)I]T4 uptake, cells were incubated in a buffer containing 142.9 mM NaCl, 4.7 PROM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.8 mM CaCl2, 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]T4 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 T2 in a final reaction volume of 300 μL. Specific D2 activity measurements were confirmed by the inhibition of deiodination reaction in the presence of T4 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 [125I]I liberated during the deiodination reaction, using a γ-counter. D2 activity was expressed as deiodinated T4 in fmol/min/mg protein. Of note is that we used 3′-5′-[125I]I T4 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]T3, 2 nM T3, 20 mM DTT, and 1 mM PTU. The addition of 200 nM of T3 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]T2 produced after reaction products separation by paper chromatography. Results were expressed as the fraction of T2 counts minus the nonspecific deiodination (always <1.5%), obtained with the saturating concentration of T3 (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**

**T₄ did not induce DC maturation and function, as T₃ does**

We demonstrated relevant findings regarding T₃ action in the initiation of adaptive immunity (Mascanfroni et al. 2008, 2010, Montesinos et al. 2012, Alamino et al. 2015). However, T₄ 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 T₄ on sensitive DC maturation/function parameters was assayed. For this purpose, murine bone marrow-derived immature DCs (iDCs) were treated with T₄ (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 T₃ (Mascanfroni et al. 2008). As shown in Fig. 1, T₄ 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 T₃ 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 T₃.

**MCT10 and LAT2 transporters are expressed in DCs and transport THs, mainly T₃**

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,
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$\left[T_3\right]$ and $^{125}$I$\left[T_4\right]$ (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$\left[T_3\right]$ was significantly higher than that of $^{125}$I$\left[T_4\right]$. 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$\left[T_3\right]$ 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$\left[T_3\right]$ uptake. As depicted in Fig. 3B, a dose-dependent reduction of $^{125}$I$\left[T_3\right]$ 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 nM, whereas the saturable $^{125}$I$\left[T_3\right]$ uptake process shows a $K_m$ value for $T_3$ inhibition of 0.76 nM and the $IC_{50}$ value for $T_4$ inhibition of $T_3$ uptake of 7.78 nM. Considering that $^{125}$I$\left[T_4\right]$ uptake was much lower than $^{125}$I$\left[T_3\right]$ uptake (Fig. 3B), the proper determination of kinetic parameters for $^{125}$I$\left[T_4\right]$ 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$\left[T_3\right]$ 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$\left[T_3\right]$ from DCs has a significantly lower velocity than $^{125}$I$\left[T_4\right]$ (Fig. 3D) as about 63% of cellular $^{125}$I$\left[T_3\right]$ remains inside DCs at 60 min after its uptake vs 32% of $^{125}$I$\left[T_4\right]$.

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$\left[T_3\right]$ uptake, whereas Leu decreased it just 15%, without significant difference from control cells. When BSP and Leu were added together, $^{125}$I$\left[T_3\right]$ uptake
Figure 3
Characterization of TH transport at DC level.
(A) Time course of 1 nM \[^{125}\text{T}_3\] (○) and \[^{125}\text{T}_4\] (●) 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 \[^{125}\text{T}_3\] at the same time of incubation. (B) Effects of increasing concentrations (1 nM–10 uM) of unlabeled iodothyronines (T3, T4) on \[^{125}\text{T}_3\] uptake in iDCs. (C) Na+ dependence of \[^{125}\text{T}_3\] uptake in DCs. Cells were incubated with 1 nM \[^{125}\text{T}_3\] 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 \[^{125}\text{T}_3\] uptake from cells incubated with Na+ containing media. (D) TH efflux from DCs. Cells were incubated for 30 min with \[^{125}\text{T}_3\]. Afterward, media was removed, cells were thoroughly washed and \[^{125}\text{T}_3\] measured at 0, 30 and 60 min thereafter. *P<0.05, **P<0.01 vs \[^{125}\text{T}_3\]. Results are expressed as mean ± S.D. of \[^{125}\text{T}_3\] uptake at 0 min and are from a representative experiment from a total of 3 with similar results performed in triplicate.

Figure 4
Effect of MCT10 and LAT2 inhibitors on T3 uptake (A) and IL-12 secretion (B) by DCs. (A) Effect of 100 uM unlabeled competitors: BSP, Leu or both on \[^{125}\text{T}_3\] uptake. DCs were incubated for 30 min with \[^{125}\text{T}_3\] 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 \[^{125}\text{T}_3\]) 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 uM competitors: BSP, Leu or both on T3-induced IL-12 secretion by DCs. DCs were incubated for 18h with T3 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 T3 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 T₃ 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 [¹²⁵I]T₃ (black bar, D3 activity) was deiodinated at a higher extent than [¹²⁵I]T₄ (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 T₃

As T₃ 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, Alamin et al. 2015), the T₃ 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 T₃ significantly increased Mct10 and Lat2 mRNAs. These results are in accordance with protein levels of both TH transporters (Western blot, Fig. 6B). In turn, [¹²⁵I]T₃ and [¹²⁵I]T₄ uptake by control iDCs and T₃-treated cells revealed that although T₃ uptake was not modified by T₃ treatment...
of iDC, T4 uptake was significantly increased 2.1-fold (Fig. 6C). On the contrary, T3 efflux was significantly increased in T3-treated DCs when compared to control iDCs (Fig. 6D), whereas T4 efflux was not modified (Fig. 6D).

Regarding T3 effect on iodothyronine deiodinase expression in DCs, T3 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 T3 treatment (1.78 fold increase vs control iDCs, Fig. 7C). Conversely, Dio2 mRNA levels tended to decrease in T3-treated DCs, but this failed to reach statistical significance (Fig. 7A). In turn, D2 protein levels were not modified by T3 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, T3, with outstanding effects at the initiation of adaptive immunity (Mascarenhas 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 (T4), 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, T4 acts mainly as a prohormone through intracellular T3 conversion to provide the major part of circulating T3 levels and to begin TH action inside target cells, although rapid nongenomic effects initiated mainly by T4 at the plasma membrane were also reported (Davis et al. 2016). The source of cellular T3, from plasma or from local T4 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 T3 (Mascarenhas et al. 2008), treatment of DCs with T4 has no effect on IL-12 production and secretion, gave us the first clue that DC response to
TH may be due, at least in its major part, directly by T₃ action. In some tissues, like the central nervous system, the T₃ derived from T₄ deiodination also acts in other cells, different from those where it was produced (St Germain et al. 2009). Therefore, although T₄ is the major circulating TH, T₃ 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 T₃ on DC characteristics and function (Mascanfroni et al. 2008, Alaino 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 T₃ 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 T₃-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 T₃ than T₄ 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 T₃ 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 T₃ is added, the saturable process shows a dose-dependent reduction in $[^{125}I]$T₃ uptake.

Figure 7
Effect of T₃ 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 T₃-treated (T₃) 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.
with an apparent $K_m$ 10 times below the concentration of $T_3$ needed to inhibit 50% of this uptake (0.76 $\mu$M vs 7.78 $\mu$M). 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′-diodothyronine ($T_2$), 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 $\mu$-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 $T_4$ 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 $K_m$ 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 $T_3$, $T_3$ 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 $T_3$ 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: rT3p, 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 $T_3$ 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 $T_3$ (Mascalfroni et al. 2008, Alamin & Tan 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 T₄ 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 T₃ 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 T₃ on the increased expression and activity of D3 was more evident than the reduction of D2 in DCs, indicate that the cellular activity of T₃ 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 T₄ and T₃ concentrations lead to homeostatic, reciprocal changes in the activity of D2 and D3. In fact, as T₃ concentrations increase, expression of Dio3 is upregulated, increasing T₃ clearance, whereas the expression of Dio2 is just modestly downregulated, decreasing T₃ production.

Worthy to be discussed, T₃ 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 T₃ 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 T₃ 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 T₃ action in vitro (Mascanfroni et al. 2010) and in vivo (Alamino et al. 2015). Altogether, T₃ 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 T₃ over T₄ 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 T₃ 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 T₃ 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.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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