Cellular Physiology and Biochemistry

Cell Physiol Biochem 2019;52:354-367
DOI: 10.33594/000000025
Published online: 28 February 2019
Accepted: 22 February 2019

Original Paper

Dendritic Cells Exposed to Triiodothyronine Deliver Pro-Inflammatory Signals and Amplify IL-17-Driven Immune Responses

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Key Words
Dendritic cells • Adaptive immunity • Triiodothyronine • Pro-inflammatory signals • Th17 • γδ-T cells

Abstract
Background/Aims: Although a cross-talk between immune and endocrine systems has been well established, the precise pathways by which these signals co-regulate pro- and anti-inflammatory responses on antigen-presenting cells remain poorly understood. In this work we investigated the mechanisms by which triiodothyronine (T3) controls T cell activity via dendritic cell (DC) modulation. ***Methods:*** DCs from wild-type (WT) and IL-6-deficient mice were pulsed with T3. Cytokine production and programmed death protein ligands (PD-L) 1 and 2 expression were assayed by flow cytometry and ELISA. Interferon-regulatory factor-4 (IRF-4) expression was evaluated by RT-qPCR and flow cytometry. The ability of DCs to stimulate allogenic splenocytes was assessed in a mixed lymphocyte reaction and the different profile markers were analyzed by flow cytometry and ELISA. For *in vivo* experiments, DCs treated with ovalbumin and T3 were injected into OTII mice. Proliferation, cytokine production, frequency of $FoxP3^+$ regulatory T (Treg) cells and $PD\text{-}1^+$ cells were determined by MTT assay, ELISA and flow cytometry, respectively. ***Results:*** T3 endows DCs with pro-inflammatory potential capable of generating IL-17-dominant responses and down-modulating expression of PD-L1 and 2. T3-stimulated WT-DCs increased the proportion of IL-17-producing splenocytes, an effect which was eliminated when splenocytes were incubated with T3-treated DCs derived from IL-6-deficient mice. Enhanced IL-17 expression was recorded in both, $CD4^-$ and $CD4^+$ populations and involved the IRF-4 pathway. Particularly, γδ-T cells but not natural killer (NK),

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NKT, B lymphocytes nor $CD8^+$ T cells were the major source of IL-17-production from $CD4^-$ cells. Moreover, T3-conditioned DCs promoted a decrease of the $FoxP3^+$ Treg population. Furthermore, T3 down-modulated PD-1 expression on $CD4^-$ cells thereby limiting inhibitory signals driven by this co-inhibitory pathway. Thus, T3 acts at the DC level to drive pro-inflammatory responses *in vitro*. Accordingly, we found that T3 induces IL-17 and IFN-γ-dominant antigen-specific responses *in vivo*. ***Conclusion:*** These results emphasize the relevance of T3 as an additional immune-endocrine checkpoint and a novel therapeutic target to modulate IL-17-mediated pro-inflammatory responses.

Introduction

Dendritic cells (DCs) are highly specialized antigen presenting cells (APC) that recognize, process, and present antigens to resting T cells for induction of antigen-specific immune responses. Moreover, they play key roles in polarization of $CD4^+$ T cells into T helper (Th) subtypes, including Th1, Th2, Th17, Th22, follicular T-helper (fTh) effector cells, as well as regulatory T cells (Treg) [1]. These $CD4^+$ T cell subsets tailor adaptive immune responses to control pathogen clearance, autoimmune and allergic processes, tissue homeostasis and antitumor responses. This specialization process is determined by innate immune microenvironments and the cytokine milieu. Whereas interferon (IFN)-γ, IFN-α, and interleukin (IL)-12 favor Th1 differentiation and IL-4 is the primary inducer of Th2 cell differentiation, transforming growth factor (TGF)-β_1 is a key cytokine required for the induction of Treg cells, but paradoxically, it can also promote pro-inflammatory responses when IL-6, IL-23 and IL-1β are present, driving the differentiation of pro-inflammatory Th17 cells [2]. Although significant progress has been made in identifying DC-derived cytokines and transcription factors leading to $CD4^+$ T cell polarization, the extracellular signals endowing DCs with specialized polarizing activity are still poorly understood.

Since the discovery and characterization of Th17 cells, several studies reported their pro-inflammatory activity in a wide variety of physiologic and pathologic settings. However, αβ-T cells do not represent the only source of IL-17; it is also produced by other cell types including γδ-T cells, natural killer (NK) cells and invariant natural killer T (iNKT) cells [3]. In addition, B cells produce IL-17 in response to challenge with infectious pathogens [4]. Moreover, a cytokine milieu containing TGF-β_1 and IL-6 may also drive the differentiation of IL-17-producing $CD8^+$ T cells (Tc17) with limited cytotoxic activity [5].

Tolerogenic mechanisms including differentiation of Treg cells, production of anti-inflammatory cytokines and expression of co-inhibitory receptors, act in concert to counterbalance pro-inflammatory signals and prevent development of autoimmune and chronic inflammatory diseases. Programmed Death protein-1 (PD-1; CD279), a co-inhibitory receptor expressed on the surface of antigen-activated and exhausted T and B cells, interacts with PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273) ligands. While PD-L1 is expressed on a wide range of non-hematopoietic cells, the expression of PD-L2 is restricted mostly to APC [6]. Interaction of PD-1 with PD-L1 triggers activation of the SHP2 phosphatases leading to inhibition of T-cell responses. This immune inhibitory checkpoint serves to maintain T-cell homeostasis by restricting T cell activation and proliferation [7]. Tumor cells co-opt PD-L1 to attenuate immune responses by triggering PD-1-dependent inhibitory signals that promote T-cell exhaustion. This inhibitory effect prompted the development of checkpoint blockade therapies by reinforcing anti-tumor T-cell responses [8].

The cross-regulation of endocrine and immune signals in the balance of pro- and anti-inflammatory signals, remain poorly understood. In this regard, we identified a critical role of triiodothyronine (T3), the most active thyroid hormone (TH), in stimulating DC maturation and function. Moreover, we found that the expression of TH receptor (TR) β1 contributes to DC maturation and Th1-type cytokine secretion induced by physiologic levels of T3 [9,10]. These effects involved activation of Akt and NF-κB pathways [10] and were counteracted

by glucocorticoids [11]. Moreover, we identified a major role of T3 in induction of cytotoxic T cells and protection against tumor development via DC activation [12, 13]. Recently, we reported the scarce effect of the main circulating TH, thyroxine (T4) on DC functionality, and described the mechanisms of TH transport across DC surface as well as TH metabolism in these cells [14].

Here, we aimed to understand the mechanisms by which TH controls T cell activity via DC modulation. In this study, we report the effects of T3-conditioned DCs in promoting the differentiation of IL-17-producing cells. These results emphasize the importance of THs as an additional immune-endocrine checkpoint and a novel therapeutic target to attenuate IL-17-mediated pro-inflammatory responses.

Materials and Methods

Mice

Wild type (WT) female C57BL/6 (B6; H-2b) and BALB/c mice were obtained from Ezeiza Atomic Center, Buenos Aires, Argentina. C57BL/6 IL-6 knockout (KO) mice (*B6.129S2- Il6tm1Kopf/*J) and C57BL/6 OTII transgenic mice (*B6.Cg-Tg (TcraTcrb) 425Cbn/J*) were from The Jackson Laboratory. Mice were maintained under specific pathogen-free conditions and used at 6–10 weeks old. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH and the local institutional animal care committee (Universidad Nacional de Cordoba).

Cell Preparation and Culture

Immature dendritic cells (iDCs) were obtained from bone marrow progenitors from C57BL/6 WT or IL-6-deficient mice as described [9]. At day 10 of culture, cells were treated with T3 (5 nM, Sigma) or lipopolysaccharides (LPS, 100 ng/ml; *E. coli* strain 0111:B4; Sigma). To rule out endotoxin contamination of T3 preparations, endotoxin content was checked (levels lower than 0.03 IU/mL, limit of detection) by the Limulus amebocyte lysate assay.

Cytokine determination

IL-6, IL-23, IL-1β, TGF-β_1, IL-17 and IFN-γ detection was performed in cell culture supernatants using standard capture ELISA (BD Biosciences PharMingen).

Flow cytometry

Cell suspensions were incubated with fluorochrome labeled-Abs for 30 min at 4°C. Different combinations of the following anti-mouse Abs (BD Biosciences, eBioscience, Biolegend) were used: PerCP-Cy5.5-conjugated anti-CD4, FITC-conjugated anti-CD8 and anti-CD3, PE-conjugated anti-CD25, APC-conjugated anti-γδ-TCR and anti-CD19, PE-Cy7-conjugated anti-NK1.1, APC-Cy7-conjugated anti-B220, PE-conjugated anti-PD-1, anti-PD-L1 and anti-PD-L2. Transcription factors Forhead box P3 (FoxP3) and interferon-regulatory factor-4 (IRF-4) were detected after cell fixation and permeabilization with FoxP3 Staining Buffer Set (eBioscience) using the following antibodies: APC-conjugated anti-FoxP3 and eFluor660-conjugated anti-IRF4 (eBioscience). For intracellular cytokine staining, DCs or allogenic cultures were exposed to brefeldin A (10 μg/ml; Sigma) for the last 4 h of cell culture. Cells were fixed and permeabilized with Cytofix/Cytoperm and Perm/Wash (BD Biosciences) and incubated with surface staining antibodies and PE-conjugated anti-IL-17 and APC-conjugated anti-IFN-γ (Biolegend). Cells were acquired on BD FACSCanto II and analyzed using the FlowJo software.

RNA Isolation and quantitative real-time RT-PCR

Total RNA purification, cDNA synthesis and quantitative PCR (qPCR) were performed as previously described [12]. Gene specific primer sets were as follow: mouse IRF-4 (150 bp), 5´-TTTATGCTTGTGCCCCACCT-3´ (forward) and 5´-TCGTCGTGGTCAGCTCTTTC-3´ (reverse); mouse β-actin (223 bp), 5´-CTACAATGAGCTGCGTGTGG-3´ (forward) and 5´-GGGCACAGTGTGGGTGAC-3´ (reverse). Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method normalized against the housekeeping gene β-actin.

Allogenic cultures

Allogenic cultures were performed to assess the ability of DCs to stimulate allogenic splenocytes *in vitro* as described [9]. Briefly, allogenic splenocytes (BALB/c, responder cells) were incubated for 3 days with irradiated DCs (C57BL/6; 30 Gy, stimulator cells) at a ratio of 1:15 (DC/splenocytes) in 96-well round-bottom plates.

In vivo model

$5x10^6$ iDCs treated with 100 μg/ml ovalbumin (OVA) and 5 nM T3 (OVA+T3-DCs) for 18 h, were injected *i.v.* into OTII transgenic mice that express an OVA-specific TCR, exhibit a 4-fold increase in peripheral T $CD4^+/CD8^+$ and T cells from lymph nodes and proliferate in an OVA dose-dependent manner [15]. One week later, splenocytes were restimulated *ex vivo* with OVA_{323} (50 ng/ml) and evaluated for proliferation (*Methyl thiazolyl tetrazolium,* MTT assay), cytokine production (ELISA), Treg frequency and PD-1 expression (flow cytometry) 4 days later.

Methyl thiazolyl tetrazolium (MTT) assay

Splenocytes were seeded in 96-well plates ($4x10^5$, 200 μl per well). Twenty μl of MTT solution (5 mg/ml) were added into each well for 4 h incubation at 37°C, and culture supernatant was discarded. Dimethyl sulfoxide (DMSO, 50 μl) was added to each well, and gently mixed with the precipitate. Optical density was measured at 540 nm (A_{540}).

Statistical analysis

Analysis of intergroup differences was conducted by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test. For the analysis of differences between two groups, the statistical significance was determined using 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

T3 endows DCs with a pro-inflammatory phenotype

Previously, we demonstrated that DCs exposed to T3 augmented IL-12 secretion, whereas production of IL-10 was not altered [9]. To further understand the precise nature of pro-inflammatory responses triggered by T3, we broaden the study of the cytokine profile induced by this treatment. As observed in Fig. 1A, IL-6, IL-23, IL-1β and TGF-β_1 were significantly increased by T3 stimulation (6.5, 84.4, 293.7 and 1.9-fold, respectively, compared with control [DC]). As a positive control, we exposed DCs to LPS which stimulated the secretion of all measured cytokines, as previously reported [16]. Thus, in addition to its role in promoting Th1 responses [9], T3 also endows DCs with the ability to produce Th17-polarizing cytokines.

The co-inhibitory ligands PD-L1 and PD-L2 are well known for their ability to dampen T-cell responses [17]. Whether TH may impact on immune checkpoint pathways is still unknown. As shown in Fig. 1B and C, T3 decreased expression levels of both, PD-L1 and PD-L2 on DCs, as evidenced by the percentage of PD-L1 and PD-L2-expressing cells (48.4% and 33.8% reduction vs control [DC], respectively). These results suggest that T3 may limit the tolerogenic capacity of DCs by favoring a pro-inflammatory cytokine profile and down-regulating PD-L1 and PD-L2 expression.

T3-stimulated DCs instruct IL-17-mediated responses

Th17 cells differentiate in the presence of pro-inflammatory cytokines including IL-6, IL-23, IL-1β in combination with TGF-β_1 [2]. Although IL-17 is considered mainly a T cell-secreted cytokine, recent evidence showed that much of the IL-17 released during an inflammatory response is produced by other immune cells [18]. Since T3 stimulates the secretion of Th17-polarizing cytokines in DCs (Fig. 1A), we then analyzed whether this hormone modulates DC ability to generate IL-17-producing T cells. For this purpose, allogenic

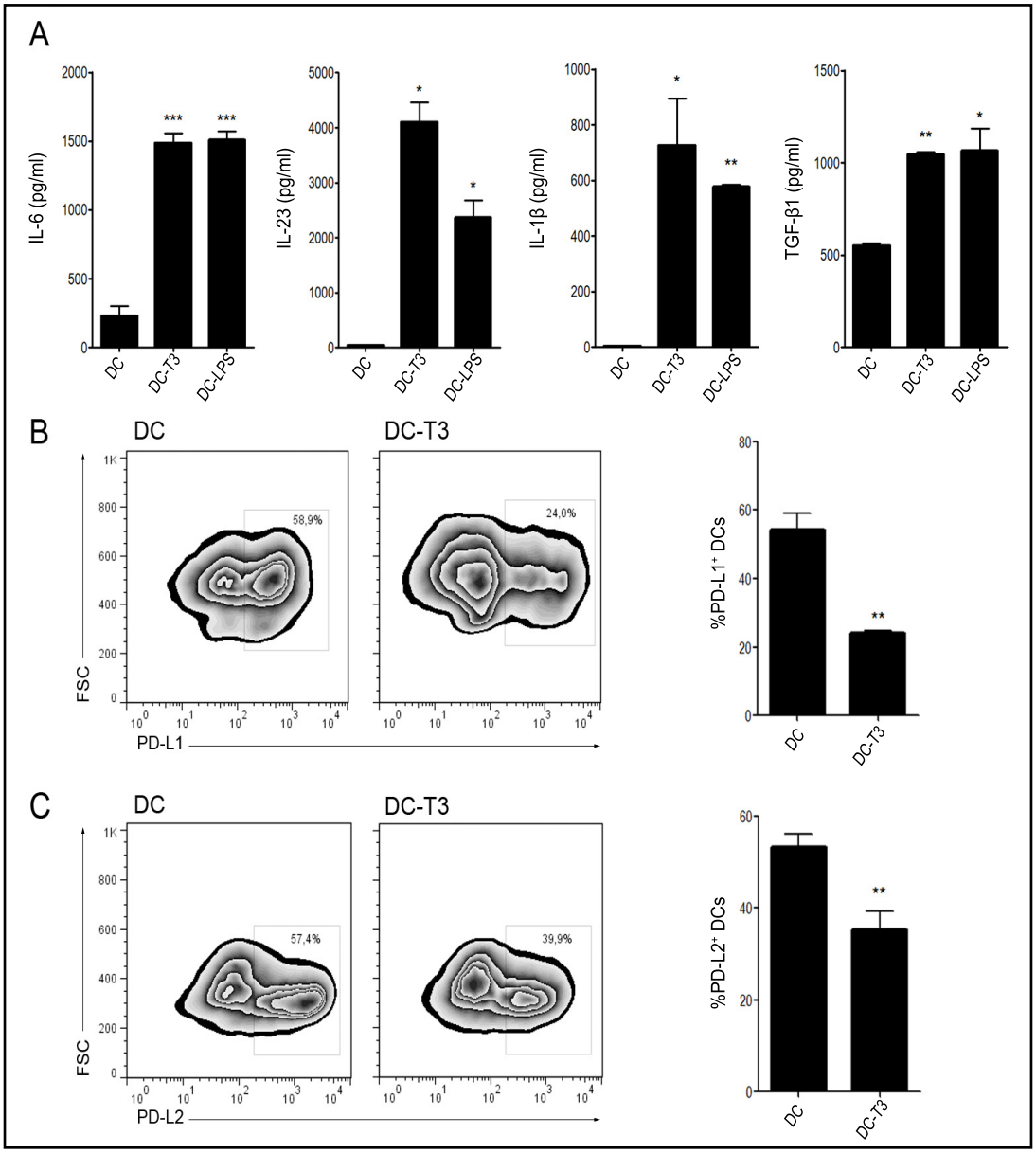

Fig. 1. Cytokine profile and PD-L1, PD-L2 expression of T3-stimulated DCs. DCs were stimulated with T3 (5 nM, DC-T3) or LPS (100 ng/ml, positive control [16], DC-LPS) for 18 h, or left untreated (DC). (A) IL-6, IL-23, IL-1β and TGF-β1 production was determined in culture supernatants by ELISA. (B, C) Expression of the co-inhibitory molecules PD-L1 and PD-L2 were determined by flow cytometry. Representative density plots of PD-L1 and PD-L2 expression (left B and C, respectively) were gated on $CD11c^+$ cells. Comparative analysis of the frequency of PD-L1 and PD-L2-expressing DCs (right B and C, respectively). Data are expressed as mean ± SD from a representative experiment of 3 with similar results performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 vs. control DC.

cultures were performed and 72 h later, IL-17 production was evaluated. As depicted in Fig. 2A-D, T3-treated DCs induced not only a higher frequency of $IL\text{-}17^+$ cells (Fig. 2A and B: 47.5% over control [DC]), but also a significant increase in intracellular IL-17 production by splenocytes (Fig. 2C: mean fluorescence intensity, MFI 58.2% vs splenocytes exposed to control [DC]), as well as higher levels of secreted IL-17 (Fig. 2D, white bars: 226.7% vs splenocytes exposed to control [DC]). Consistent with the critical role of IL-6 in driving Th17 responses [19], T3-treated DCs obtained from IL-6-deficient mice co-cultured with allogenic

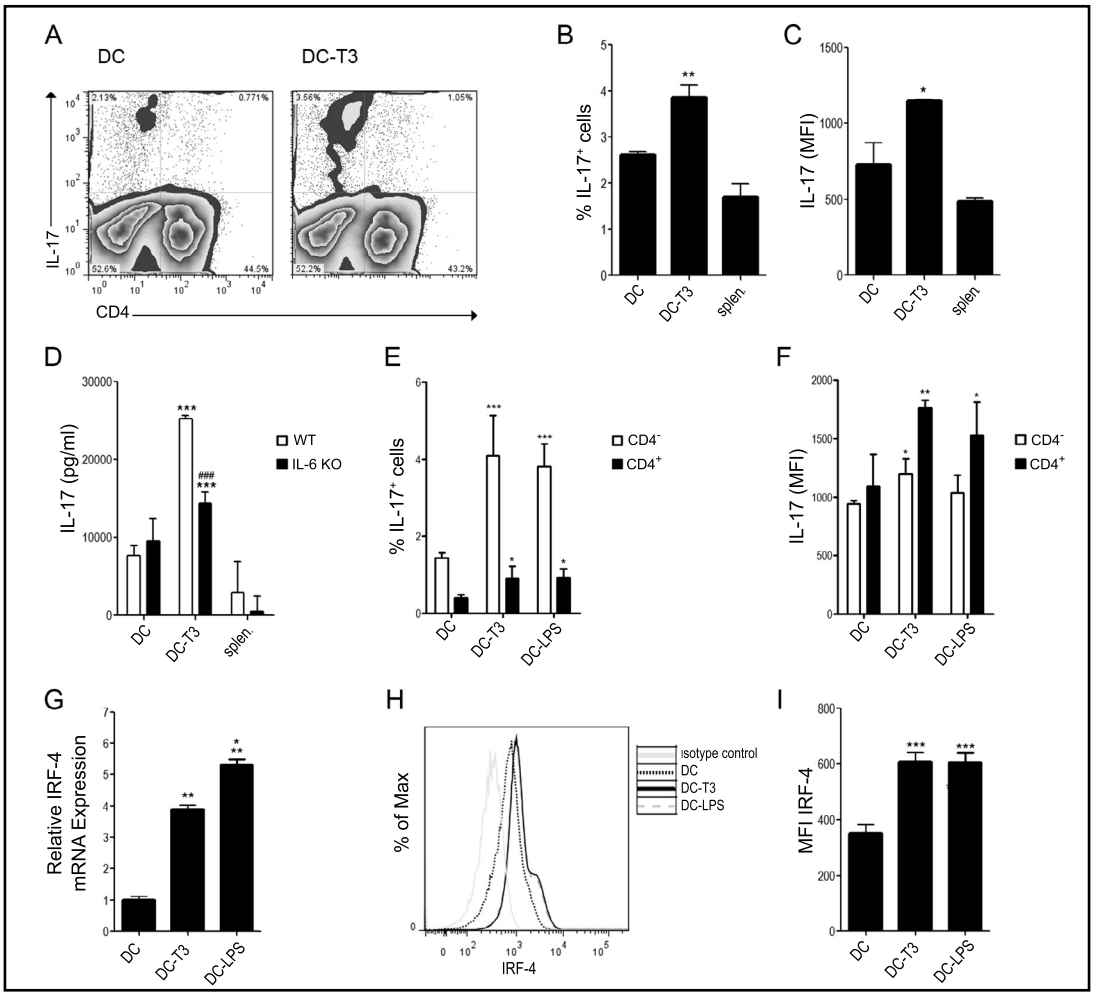

Fig. 2. T3-stimulated DCs induce IL-17 production. DCs obtained from wild type (WT) or IL-6 knockout (KO) mice were stimulated with T3 (5 nM, DC-T3) or LPS (100 ng/ml, DC-LPS) for 18 h or left untreated (DC). Then, DCs were cultured for 3 days with allogenic splenocytes (splen.). Intracellular IL-17 production was measured by flow cytometry (A, B, C, E, F) and in culture supernatants by ELISA (D). Values in representative density plots show the percentage of activated $CD4^+$ and $CD4^-$ T cells producing IL-17 (A). Comparative analysis of the frequency (B) and mean fluorescence intensity (MFI, C) of total IL-17 expressing cells. Determination of IL-17 secretion in culture supernatants from allogenic cultures with WT or IL-6 KO DCs (D). Comparative analysis of the frequency (E) and MFI (F) of $CD4^+$ and $CD4^-$ IL-17 expressing cells. IRF-4 mRNA levels measured by RT-qPCR (G) and IRF-4 intracellular protein by flow cytometry: expressing cells (H, I). Results are expressed as mean ± SD of a representative experiment of 3 with similar results performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 vs. DC; ***p<0.001 vs. DC WT or KO, respectively; ###p<0.001 vs. DC-T3 WT.

WT splenocytes secreted considerably lower levels of IL-17 as compared to control DCs (Fig. 2D, black bars: 51.3% over control [DC]). Thus, IL-6 contributes to the Th17 polarizing activity of T3-conditioned DCs.

Interestingly, increased IL-17 production by splenic cells exposed to T3-stimulated DCs (Fig. 2A) was evident in both $CD4^-$ and $CD4^+$ cells (Fig. 2A, E and F). In this regard, $CD4^-$ and $CD4^+$ $IL\text{-}17^+$ cells significantly increased their frequency (Fig. 2E, $CD4^-$: 184% and $CD4^+$: 123% over control [DC]) and intracellular levels of IL-17 (Fig. 2F, $CD4^-$: 27% and $CD4^+$: 61% over control [DC]) after co-culture with T3-treated DCs compared to control DCs. Notably, results from Fig. 2A and E show that the main source of IL-17 production was the $CD4^-$ population. However, an increase of IL-17-producing $CD4^+$ cells was also found when we cultured

splenocytes with T3-conditioned DCs (Fig. 2A and E). A comparable result was obtained in the frequency of Th17 cells, evaluated by co-culture of purified allogenic and syngenic $CD4^+$ cells with DCs stimulated by T3 (data not shown). This result suggests that different cell types within the splenocyte population may contribute to IL-17 production induced by T3-treated DCs.

IRF-4 is a transcription factor closely related to IL-17-mediated responses [20, 21]. Interestingly, T3-stimulated DCs showed enhanced expression of IRF-4 mRNA and intracellular protein (Fig. 2G, 4.5, and Fig. 2H and I; 0.7 fold increase over control [DC], respectively). These results show that T3-stimulated DCs direct the differentiation of IL-17-producing $CD4^-$ and $CD4^+$ T cells through an effect accompanied by higher IRF-4 mRNA and protein expression.

γδ-T cells are the main source of IL-17 induced by T3-stimulated DCs

Given that increased IL-17 production induced by T3-stimulated DCs is mainly driven by a $CD4^-$ population, further phenotypic analysis of this subset was conducted. As shown in Fig. 3, T3-stimulated DCs induced a significant increase in the frequency of IL-17-expressing γδ-T cells ($CD3^+$, γδ-TCR^+, Fig. 3A, DC vs DC-T3: 33% vs 40%). Neither NK ($CD3^-$, $NK1.1^+$; Fig. 3B) nor NKT ($CD3^+$, $NK1.1^+$; Fig. 3B) cells showed significant modifications in IL-17 secretion. Of note, the recorded decrease in the percentage of $IL\text{-}17^+$ B cells ($CD19^+$, $B220^+$; Fig. 3C) could result from the increase in $IL\text{-}17^+$-expressing γδ-T cells, as the absolute number was not altered (data not shown).

To explore the possible contribution of Tc17 to increased IL-17 production induced by T3-conditioned DCs, we further evaluated $CD8^+$ cells as a source of this cytokine. As shown in Fig. 3.D, Tc17 cells were almost absent within the splenocyte population co-cultured with either control or T3-stimulated DCs. Thus, γδ-T cells are a major source of IL-17 induced by T3-educated DCs.

T3-stimulated DCs restrain Treg cell differentiation and induction of $PD\text{-}1^+$ $CD4^-$ T cells

To further understand the differential regulation of tolerogenic and immunogenic circuits triggered by T3-stimulated DCs, we then analyzed their influence on the Treg cell compartment. Results revealed a significant reduction in the frequency of $CD25^+$, $FoxP3^+$ Treg cells when splenocytes were co-cultured with T3-stimulated DCs compared with control DCs (DC-T3: 24% under DC; Fig. 4A, right panel). Along with the induction of $IL\text{-}17^+$ cells (Fig. 2 and 3) and Th1 cells [9], these findings support the notion that T3 acts at the DC level to drive pro-inflammatory responses.

Next, we evaluated the capacity of T3-treated DCs to modulate the expression of PD-1 on T cells. As shown in Fig. 4B, PD-1 expressing cells were significantly reduced when the $CD4^-$ population was exposed to T3-treated DCs (Fig. 4B, left and middle panel, frequency: 33% of control value) with a tendency towards reduction in $CD4^{high}$ cells (Fig. 4B, left and right panel). In accordance, the MFI of PD-1-expressing cells was also lower in the $CD4^-$ population (data not shown). Surprisingly, PD-1 expression on $CD4^-$ cells did not correspond to the CD8 population since the frequency of $PD\text{-}1^+$ $CD8^+$ cells was not altered by co-culture with T3-stimulated DCs (Fig. 4C). This result suggests that T3-conditioned DCs could limit PD-1 expression on $CD4^-$ γδ-T cells as well as other $CD4^-$ cells. Thus, T3 counteracts immune inhibitory pathways through modulation of DC function.

T3 tilts the balance towards a pro-inflammatory T cell response in vivo

Given the effects of T3-stimulated DCs in development of pro-inflammatory responses *in vitro*, we then examined their pathophysiologic relevance *in vivo*. For this purpose, OTII transgenic mice that express an OVA-specific TCR were used as an antigen-specific experimental model. DCs from syngeneic mice pulsed with OVA and treated with T3 (OVA+T3-DC) for 18 h, or not (control, OVA-DC), were injected *i.v.* into OTII transgenic mice. One week later, splenocytes were isolated and restimulated *ex vivo* with OVA_{323} peptide. We found a significant increase in the proliferation of splenocytes from mice inoculated with

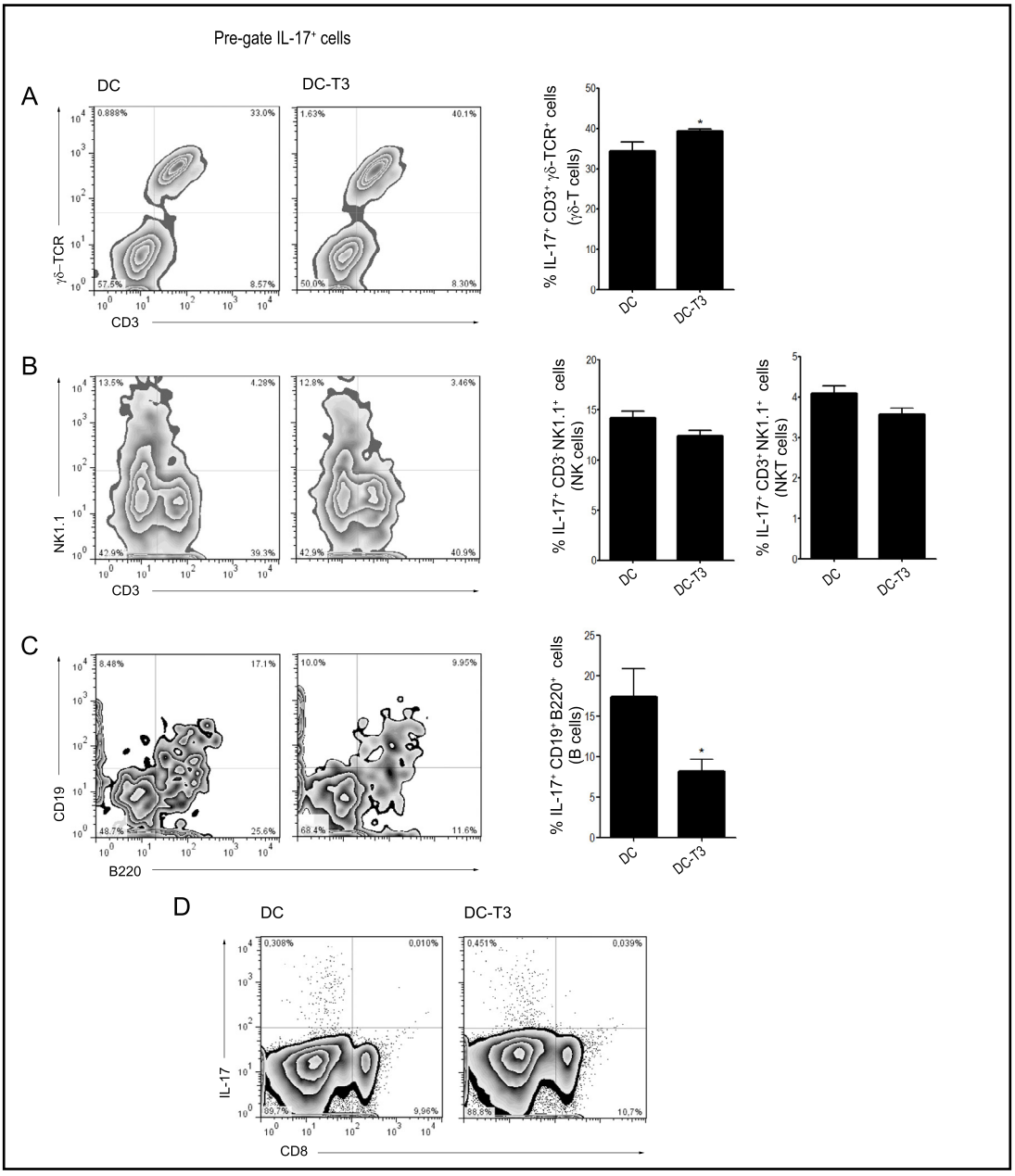

Fig. 3. Characterization of the IL-17-producing $CD4^-$ population. DCs were stimulated with T3 (5 nM, DC-T3) for 18 h or left untreated (DC) and then cultured with allogenic splenocytes for 3 days. The characterization of cell populations was determined by their specific markers by flow cytometry. γδ-T cells ($CD3^+$, γδ-TCR^+), NK cells ($CD3^-$, NK 1.1^+), NKT cells ($CD3^+$, NK 1.1^+), B cells ($CD19^+$, $B220^+$) and $CD8^+$ T cells ($CD8^+$). Representative density plots of γδ-T, NK, NKT and B cell subsets analysis are gated on IL-17 producing $CD4^-$ T cells (left A, B and C, respectively). Comparative analysis of frequency of IL-17 expressing γδ-T, NK, NKT and B cells (right A, B and C, respectively). (D) Analysis of IL-17-producing $CD8^+$ T cells. Results are expressed as mean ± SD from a representative experiment of 3 with similar results performed in triplicate. *$p<0.05$ vs. DC.

OVA+T3-DC in the absence (Fig. 5A, white bars, 3.3-fold vs OVA-DC) or in the presence (Fig. 5A, black bars, 5.2 times vs OVA-DC) of OVA_{323} restimulation. In turn, IL-17 and IFN-γ levels were increased in culture medium from OVA+T3-DC vs OVA-DC (Fig. 5B and E, white bars). Moreover, after restimulation with $OVA_{323,}$ both IL-17 and IFN-γ augmented in medium from splenocytes isolated from mice inoculated with T3-conditioned, OVA-pulsed DCs (Fig 5B and

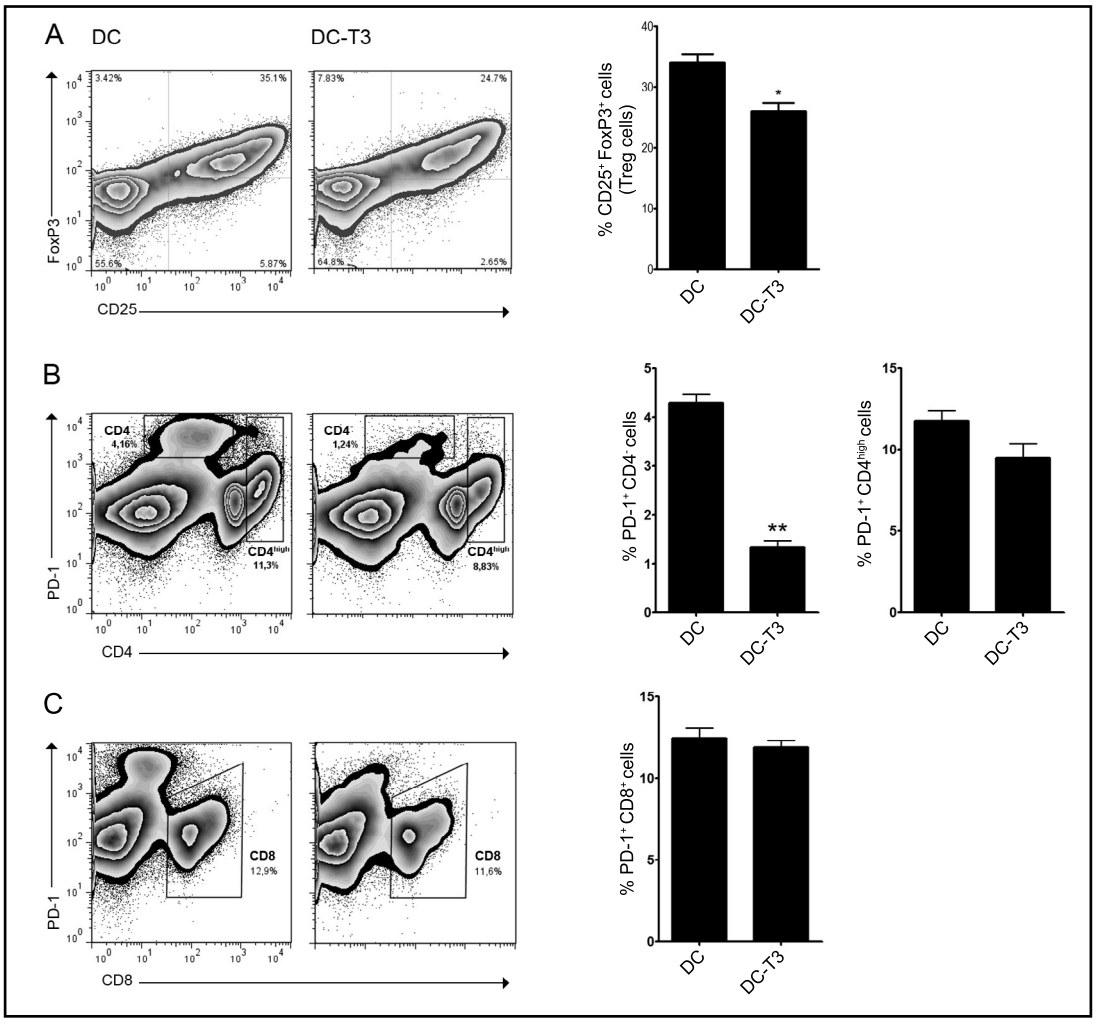

Fig. 4. Effect of T3-stimulated DCs on Treg cells and PD-1-expressing CD4 and CD8 cells. DCs were stimulated with T3 (5 nM, DC-T3) or left untreated (DC). After 18 h, they were cultured with allogenic splenocytes for 3 days. (A) $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells were determined by flow cytometry. Values in representative density plots show the percentage of $CD25^+$, $FoxP3^+$ in $CD4^+$ gated cells (A, left panel). Comparative analysis of $CD25^+$ $FoxP3^+$ cells within the $CD4^+$ T cell population (A, right panel). (B, C) Expression of PD-1 on CD4 and CD8 cells was determined by flow cytometry. (B, C) Representative density plots show the percentage of PD-1 expressing $CD4^-$, $CD4^{high}$ and $CD8^+$ cells (B, C, left panel). Comparative analysis of cellular frequency (B, C, right panel). Data are expressed as mean ± SD and are representative of 3 experiments with similar results performed in triplicate, *p< 0.05, **p<0.01 vs control DC.

E, black vs white bars OVA+T3-DC group: 2.1-fold and 2.0-fold, respectively). In addition, an increase in IL-17-producing $CD4^+$ T cells was recorded in the OVA+T3-DC group after restimulation with OVA_{323} (Fig. 5C, black vs white bars, 1.5-times over unstimulated cells). Of note, although the rise in IL-17^+ $CD4^-$ cells did not reach statistical significance in the OVA+T3-DC group, there was a tendency towards an increase when compared to control mice treated with OVA-DC not exposed to T3 (Fig. 5D). Besides, and in agreement with IL-17 released, IFN-γ^+-secreting $CD4^+$ and $CD8^+$ cells increased in the spleens from mice inoculated with OVA+T3-DC vs OVA-DC (Fig. 5F and 5G, respectively), although $CD4^+$ cells increased only after restimulation with OVA_{323}. In turn, the frequency of IL-17/IFN-γ double-positive cells was very low in both the $CD4^-$ and $CD4^+$ populations (data not shown).

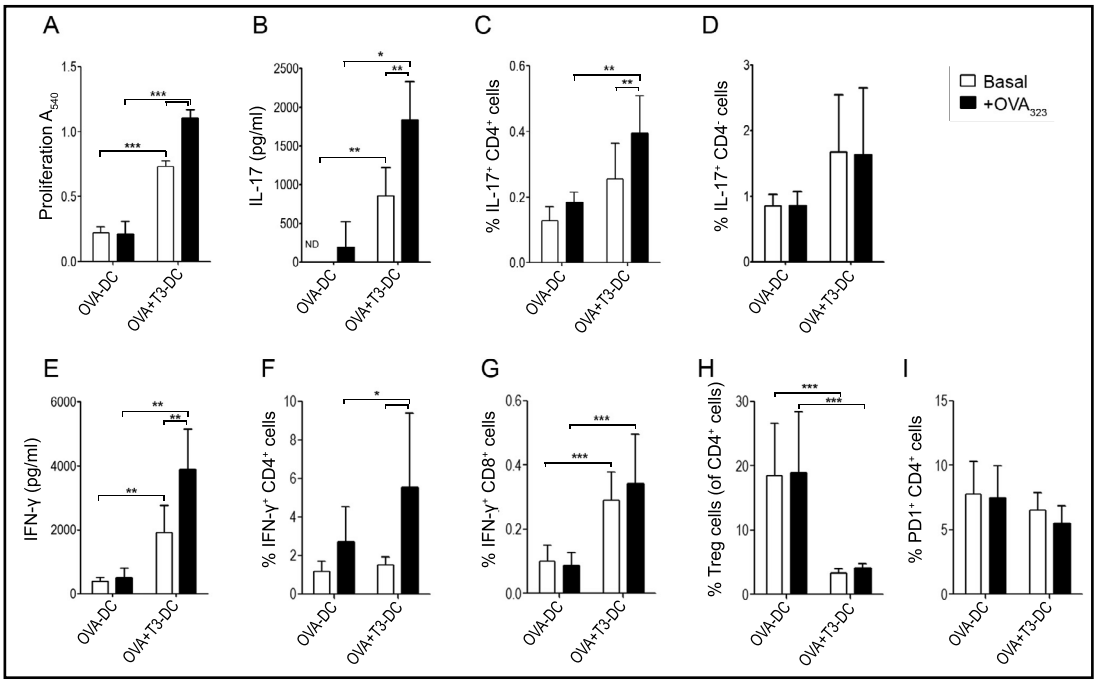

Fig. 5. T3-stimulated DCs induced a pro-inflammatory response *in vivo*. DCs treated with ovalbumin (100 µg/ml, OVA-DC) and 5 nM T3 (OVA+T3-DC) for 18 h, were injected i.v. into syngenic OTII transgenic mice. One week later, spleen cell suspensions were prepared and cultured with OVA_{323} peptide (50 ng/ml), and proliferation (A), IL-17A (B, C, D) and IFN-γ production (E, F, G), $CD4^+CD25^+FoxP3^+$ Treg cells (H) and PD-1-expressing CD4 cells (I) were determined 4 days later. Data are expressed as mean ± SD and are representative of 3 experiments with similar results performed in triplicate (4 mice per group). *p<0.05, **p<0.01, ***p<0.001.

Finally, the regulatory response induced by T3-treated DCs was also explored *in vivo.* Supporting the *in vitro* observations, we found a reduction of Treg cell frequency in spleens from OTII mice inoculated with T3-conditioned, OVA-pulsed DCs as evidenced by a reduction in $CD4^+$ $CD25^+$ $FoxP3^+$ cells, both in basal conditions and following restimulation with OVA_{323} (Fig. 5H, basal: 5.6-fold and after OVA_{323}: 4.6-fold from OVA-DC values, respectively). In addition, when mice were injected with OVA+T3-DC, a tendency towards a reduction in PD-1 expression on $CD4^+$ cells was recorded (compared with splenocytes from mice treated with DC-OVA; Fig. 5I), particularly after restimulation with OVA_{323}, without reaching statistical significance.

Thus, T3 endows DCs with the capacity to promote both IFN-γ- and IL-17-mediated antigen-specific responses and down-modulate Treg cells *in vivo*.

Discussion

Thyroid hormones play key roles linking immune and endocrine networks. Our laboratory has centered the attention in elucidating the role of T3 in the initiation and termination of adaptive immune responses with a special focus on its role within the DC compartment [9-12, 14]. Although an initial therapeutic impact of T3-conditioned DCs was revealed *in vivo* in cancer settings [12, 13], the precise characterization of the immune landscape modulated by these cells is still lacking.

Our results demonstrated that splenocytes co-cultured with T3-matured DCs secreted higher levels of IL-17. This finding correlated with increased production of Th17-promoting cytokines: IL-23, IL-1β, IL-6 and TGF-β_1 by T3-stimulated DCs. These results integrate

with our previous findings demonstrating that T3 endows DCs with enhanced ability to polarize Th1 responses *in vitro* [9], as well as cytotoxic and antitumor effects *in vivo* [12, 13]. Accordingly, *in vivo* studies revealed the ability of T3-conditioned DCs to promote IFN-γ and IL-17-dominant antigen-specific adaptive responses. Induction of simultaneous Th1 and Th17 responses were also reported for DCs stimulated by resuscitation-promoting factor (Rpf) E of *Mycobacterium tuberculosis* leading to production of pro-inflammatory cytokines IL-12, IL-1β, IL-6, IL-23 and TNF and instructing $CD4^+$ T cells to secrete IFN-γ, IL-2 and IL-17 [22]. A similar pattern of Th cytokine production was also observed in co-cultures of $CD4^+$ T cells with DCs treated with Loxoribine, a selective Toll-like receptor (TLR)-7 agonist [23].

Although Th1 cells have shown broad antitumor activity [24] and T3-conditioned DCs increase IFN-γ-producing CD8 responses in cancer [12, 13], the role of Th17 and other IL-17-producing cells in neoplastic settings is controversial. A growing body of evidence supports the notion that Th17 cells have pro-carcinogenic activity during inflammation-induced tumorigenesis [25]. However, it was reported that IL-17 and Th17 cells have both tumor-promoting as well as tumor-suppressing functions, and part of this controversy arose when different aspects of the IL-17 response were studied [26]. In this regard, a connection has been proposed between DC activation, their recruitment to the tumor microenvironment and the T helper cytokine profile. In humans, IL-17 produced by macrophages and Th17 cells were associated with high microvessel density and poor survival in colorectal carcinoma [27], whereas mast cells expressing IL-17 indicate a favorable prognosis in esophageal squamous cell carcinoma [28]. Of note, vaccination of melanoma patients with tumor lysate-pulsed DCs increases both Th1 and Th17 cells in the peripheral blood and this effect correlated with prolonged survival [29]. Interestingly, whereas IL-17 mainly promoted tumorigenesis, a subpopulation of IL-17-producing Th17 cells seems to have tumor-suppressing effects, emphasizing the importance of the IL-17 source as well as the type of tumor in delineating the function of this cytokine [26]. In this regard, recent studies highlighted the double-edge effects of cytokines acting either as anti- or pro-tumorigenic agents depending on the tumor type [30]. Notably, consistent with the increased $CD8^+$ T-cell proliferation and antigen-specific cross-presentation induced by T3-exposed DCs [12] and the enhanced IL-17 production by γδ-T cells (this work), previous studies showed a central role for IL-17A-producing γδ-T cells during infection [31]. Moreover, given the paradoxical pathogenic and regulatory roles of IL-17-producing cells [32], further studies should be aimed at investigating the potential role of T3-conditioned DC in autoimmune disorders. Besides, the very low splenic $CD4^+$ IFN-γ-IL-17 double-positive cells induced by T3-treated DCs disregards a major contribution of canonical pathogenic Th17 cells to this effect [33].

In turn, results revealed that T3-exposed DCs also promote expansion of IL-17-expressing γδ-T cells, which play an ambiguous role in antitumor immunity [34]. By contributing to IL-17A and IFN-γ secretion, these cells display cytotoxic functions in a broad range of tumor cells, including prostate cancer, melanoma, metastatic renal carcinoma, breast and ovarian cancer, colon carcinoma, hepatocellular carcinoma, lung cancer and myeloma [35]. Besides, a protective role of γδ-T cells in multiple mouse cancer models has been established *in vivo*, largely on the basis of their potent cytotoxic activity and IFN-γ production. These cells secrete substantial amounts of IFN-γ, which inhibits angiogenesis and enhances MHC class I expression by tumor cells, thus promoting $CD8^+$ T cell responses [36]. IL-17-producing γδ-T cells promote recruitment of conventional Th17 cells, which in turn activate $CD8^+$ T cells through IFN-γ-dependent mechanisms. Effectors Th1, Th17 and $CD8^+$ T cells mediate antitumor activity and inhibit tumor growth. On the contrary, regulatory γδ-T (γδ-Treg) cells promote tumor growth by impairing the function of effector T cells [35]. Interestingly, in response to commensal microbiota and pro-inflammatory cytokines, γδ-Treg cells amplify immunosuppressive circuits by secreting high amounts of galectin-1, an endogenous immunoregulatory lectin [37].

Treg cells play a central role in the control of autoimmunity, tissue homeostasis, microbial infection and tumor growth [28]. In malignant tumors, these cells promote tumor progression by suppressing effective antitumor immunity. High infiltration of Treg cells in

tumors is associated with a poor prognosis in various cancer types [38]. Thus, stimulation of Th1/Th17 responses at the expense of contraction of Treg cell compartment in mice receiving T3-conditioned DCs could influence the clinical outcome of DC-based antitumor vaccines [12, 13].

In this regard, our findings also demonstrate the influence of T3 in modulating the PD-1/PD-L1 axis. We found that T3 reduced the expression of PD-L1 and PD-L2 on T3-conditioned DCs. In turn, T3-conditioned DCs trigger a decrease in the frequency of PD-1-expressing T cells both *in vitro* and *in vivo*. As immune checkpoint blockers are essential components of the arsenal of immunotherapeutic drugs [39], our results highlight the relevance of T3-conditioned DC vaccines as a possible complement to augment the clinical efficacy of anti-PD-1/PD-L1 therapy. These findings may also provide a rational explanation for the antitumor effects of T3-stimulated DC vaccination in B16 melanoma [12, 13], decreasing PD-L1, PD-L2 or PD-1 expression in the tumor microenvironment.

Conclusion

Taken together, the results presented in this work reinforce the critical role of T3 in the initiation of adaptive immunity and induction of pro-inflammatory responses. Thus, T3, at physiologic concentrations, provides a novel opportunity for manipulating the immunogenic potential of DCs to positively regulate the development of antimicrobial and antitumor immunity and limit the occurrence of autoimmune inflammation. These findings underlie the intimate connections between immune and endocrine systems in the modulation of inflammatory responses.

Acknowledgements

The authors thank Laura Gatica, Gabriela Furlan, Paula Abadie, Pilar Crespo and Alejandra Romero for technical assistance in flow cytometry analysis and cell culture, and Luis Navarro, Fabricio Navarro, Diego Luti, Victoria Blanco and Carolina Florit for helping in animal care. This study was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SeCyT) and Fundación Sales.

Disclosure Statement

The authors disclose no potential conflicts of interest.

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