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FICZ generates human tDCs that induce CD4$^+$ CD25^{high} Foxp3$^+$ Treg-like cell differentiation

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ABSTRACT

Dendritic cells (DCs) play a central role in the maintenance of immune homeostasis, their participation as professional antigen presenting cells is essential to the initiation of the adaptive immune response as well as to the induction of tolerance. The recently described role of the aryl hydrocarbon receptor (AhR) in the immune system, particularly in the modulation of the adaptive immune response has attracted the attention as a potential player in the induction of immune tolerance. However, the effects of AhR activation through endogenous ligands on human DCs have been poorly evaluated. In this study, we investigated the effect of FICZ, a natural AhR ligand, on monocyte-derived dendritic cells (Mo-DCs) from healthy subjects. We found that the activation of AhR through FICZ during DCs differentiation and maturation processes resulted in a decreased expression of CD83, an increased expression of the enzyme IDO and a reduced production of the pro-inflammatory cytokines IL-6 and TNF-α. More importantly, FICZ-treated DCs were able to induce the differentiation of naive T lymphocytes into CD4$^+$ CD25^{high} Foxp3$^+$ T reg-like cells. Our results show that the activation of the AhR on human DCs induces a tolerogenic phenotype with potential implications in immunotherapy.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APC) that play important roles in initiating effective adaptive immune responses for elimination of invading pathogens and also in inducing immune tolerance toward self-antigens and commensal organisms, to maintain immune homeostasis. The disruption of the fine balance between tolerance and immunity mediated by DCs can lead to chronic inflammation, susceptibility to infections, cancer progression and autoimmune disease development [1–4].

Within the heterogeneous population of DCs, the subset of tolerogenic DCs (tDCs) had received special attention due to its role in modulating the immune response [5]. Regulatory properties of DCs depend on different factors including maturation stage, microenvironment cues, interaction with anti-inflammatory and immunosuppressive molecules and the nature of certain pathogen components [6,7]. tDCs carry out their function by expressing immunomodulatory molecules (e.g. PD-L1, CTLA-4, ICOSL, OX40L and ILT3) and producing immunosuppressive factors (IL-10, TGF-β, indoleamine 2, 3-dioxigenase and NO). These cells promote immunologic tolerance through a variety of mechanisms that include: deletion of pathogenic T cells, induction of anergic T cells and generation of Treg cells [8–10]. The recent approach on the use of tDCs in the treatment of immune-mediated diseases such as autoimmunity, allergies and allograft rejection, has led to a special interest in the search for new compounds, signaling pathways or strategies that can induce tolerogenic properties on DCs [11–14].

The aryl hydrocarbon receptor (AhR) was discovered 30 years ago as a mediator of toxic response to environmental pollutants [15]; however, its involvement in additional processes such as reproduction, circadian rhythm, neurotransmission, cell cycle, among others, has recently been established [16–19]. The AhR is a ligand-activated transcription factor member of the basic helix-loop-helix Per-Arnt-Sim

Abbreviations: DCs, dendritic cells; tDCs, tolerogenic dendritic cells; AhR, aryl hydrocarbon receptor; Tregs, regulatory T cells; IDO, indoleamine 2,3, dioxygenase; FICZ, 6-formylindolo (3,2-b)carbazole; TNF-α, tumor necrosis factor alpha; PGE2, prostaglandin E2; iDCs, immature dendritic cells; mDCs, mature dendritic cells; Mo-DCs, monocyte-derived dendritic cells; CFSE, carboxyfluorescein diacetate succinimidyl ester

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(bHLH-PAS) protein family. In absence of ligand, AhR is located in an inactive state as part of a cytoplasmic protein complex containing heat shock protein 90 (HSP 90), AhR-interacting protein (AIP) and p23. Ligand binding to the AhR, triggers a conformational change allowing exposure of its nuclear localization sequence and subsequent translocation to the nucleus, where the complex dissociate and AhR dimerizes with the AhR nuclear translocator (ARNT). The AhR-ARNT heterodimer finally binds to specific DNA sequences termed dioxin-response elements (DRE), thereby regulating expression of target genes, among theme those encoding enzymes belonging to the cytochrome P450 family, which are essential for the metabolism of xenobiotics [15,20].

The AhR is widely expressed in immune cells and currently considerable evidence indicates that it plays an important role in the immune response. Several studies have reported a key role of the AhR on differentiation of T lymphocyte subsets such as Th17 and Treg [21–23]. A variety of ligands from different nature (endogenous metabolites, dietary compounds, microbial derivatives and xenobiotics) have shown their ability to activate the AhR and modulate the expansion of Tregs and Th17 cells in models of arthritis, delayed-type hypersensitivity, experimental autoimmune encephalomyelitis, ulcerative colitis, and bacterial infection, among others [24–28]. Likewise, the potential role of the AhR in the innate immunity has been gradually explored, revealing an important but poorly known side of the function of this receptor [20,29,30].

Bridging innate and adaptive immunity, DCs have been described as targets of the AhR-mediated immune suppression through mechanisms that are gradually being elucidated [29–31]. It has been described that murine DCs treated with the natural AhR ligands indole-3-carbinol (IC3) and indirubin-3′-oxime (IO), showed decreased expression of CD11c, CD40 and CD54. In addition, production of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IL-12 and nitric oxide are suppressed whereas levels of the anti-inflammatory cytokine IL-10 are increased in the presence of AhR ligands. Moreover, an increased expression of some regulatory genes including retinaldehyde dehydrogenase 1 (ALDH1A1), TGF-β2, TGF-β3, IDO1 and IDO2 has been observed in DCs after AhR activation [32]. All these findings suggest the participation of AhR in several processes involved in the induction of tolerogenic DCs [26,33]. This field continues to expand due to the discovery of new AhR ligands, including: tryptophan metabolites (e.g. kynurenine, FICZ, ITE), indoles generated by bacterial metabolism and dietary ligands from cruciferous vegetables (e.g. I3C, ICZ) [20,29]. Currently, the attention has been attracted to exploring promising endogenous ligands of AhR for the generation of tDCs for potential clinical applications, without the side effects caused by exogenous ligands. Therefore, the aim of the present study was to evaluate the effect of the natural AhR ligand FICZ on human DCs.

2. Materials and methods

2.1. Generation of monocyte-derived dendritic cells (Mo-DCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy subjects by Ficoll-Paque density gradient centrifugation (GE Healthcare, Wilmington, MA, USA). $CD14^+$ monocytes were isolated from the PBMCs by positive selection using anti-CD14 mAbs coupled to Microbeads, according to manufactureŕs directions (Miltenyi Biotec, San Diego, CA, USA). The purity of isolated monocytes was verified by flow cytometric analysis and was always higher than 90%. Monocytes were cultured at 1×10^6 cells/mL in RPMI 1640 culture medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2.0 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA) and 50 mM 2-mercaptoethanol (Gibco), in the presence of 200 ng/mL recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) and 15 ng/mL recombinant human interleukin-4 (rhIL-4) (PeproTech, Rocky Hill, NJ, USA), in an atmosphere of 5% CO_2 at 37 °C. Additionally, 50 or 300 nM FICZ (Enzo Life Sciences, Farmingdale, NY, USA) or vehicle control (< 0.1% DMSO) (Sigma), was added to the culture medium. rhGM-CSF, rhIL-4, FICZ and culture medium were refreshed on days 2 and 4, keeping the same concentrations. On day 6, immature DCs were harvested and reseeded during 48 h in the presence of 40 ng/mL TNF-α (PeproTech) and 0.35 μg/mL PGE2 (Sigma) to induce their maturation, keeping conditions and treatment (FICZ or vehicle control) they had during the differentiation process. Supernatants from immature and mature DCs were collected and stored at −60 °C for cytokine quantification.

2.2. Analysis of DC and T cell phenotype by flow cytometry

Phenotype of iDCs and mDCs was analyzed by staining the cells with the following anti-human monoclonal antibodies (mAbs): CD11c-APC, CD83-PE (eBioscience, San Diego, CA, USA), HLA-DR-APC, CD80-PE-Cy7 and CD86-PerCP-Cy5.5 (BioLegend, San Diego, CA, USA) for 20 min at 4 °C. Cells were acquired on a BD FACSCanto II flow cytometer (Becton Dickinson, San José, CA, USA) and analyzed using the FlowJo v7.6.5 software (Tree Star, Ashland, OR, USA). To analyze AhR and IDO expression in iDCs and mDCs, cells were stained with anti-CD11c-APC for 20 min at 4 °C. Then, cells were fixed and permeabilized using the Foxp3 Fix/Perm kit (eBioscience) and stained at intracellular level with mAbs anti-AhR-PE (eBioscience) or anti-IDO-PE (eBioscience) for 30 min at 4 °C. Finally, cells were acquired and analyzed as mentioned above.

2.3. Cytokine quantification

IL-12p70, IL-6, TNF-α and IL-1β production was quantified in cell culture supernatants by BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (Becton Dickinson) according to manufactureŕs instructions. The CBA analysis was performed on a BD FACSCanto II flow cytometer (Becton Dickinson) using FCAP Array v3.0 software (Soft Flow, St. Louis Park, MN, USA).

2.4. DC-T cell co-cultures

Two samples of peripheral venous blood were collected from the same donor at two different time points. The first blood sample was collected on day 0, in order to perform the separation of $CD14^+$ monocytes and induce their differentiation into Mo-DCs in the presence of vehicle or 300 nM FICZ, as described in Section 2.1. The second blood sample was collected on day 8 in order to isolate naive T cells by using the EasySep ™ Human Naïve $CD4^+$ T Cell Enrichment Kit according to manufactureŕs directions (STEMCELL Technologies Inc., Vancouver, BC, Canada). For the induction of Treg differentiation, autologous mature Mo-DCs were harvested on day 8, washed twice with PBS solution and then co-cultured with purified naive $CD4^+$ T cells at a 1:10 ratio, in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) and 2.0 mM L-glutamine (Sigma), in 48-well plates previously coated with 5 μg/mL anti-CD3 and 5 μg/mL anti-CD28 (BioLegend). Co-cultures were incubated during four days in an atmosphere of 5% CO_2 at 37 °C, and at the end of this period of time, cells were harvested and stained with anti-CD4-FITC (Becton Dickinson) and anti-CD25-PE (eBioscience) mAbs for 20 min at 4 °C. Thereafter, cells were permeabilized and stained with an anti-Foxp3-APC mAb (eBioscience) for 30 min at 4 °C. Finally, cells were acquired on a BD FACSCanto II flow cytometer (Becton Dickinson) and analyzed using the FlowJo v7.6.5 software (Tree Star). As a positive control, Treg polarization was performed in the presence of 50 U/mL IL-2 (eBioscience) and 10 nM TGF-β (PeproTech).