NEW INSIGHT INTO THE BIOLOGICAL EFFECT OF TCDD ON CD4$^+$ HELP T CELL: NOT ONLY IMMUNOTOXICITY BUT ALSO IMMUNE REGULATION

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Introduction

Accidental acute exposure to 2,3,7,8-tetrachloodibenzo-p-dioxin (TCDD) is associated with the increase in a variety of human health problems, including immune dysfunction, neurological pathologies, abnormal development, diabetes, and carcinogenesis[1]. Previous and traditional toxicological studies have recognized the immune system as a sensitive target of TCDD and related halogenated aromatic hydrocarbons. It is recognized that suppression of the immune system is one of the most sensitive endpoints of TCDD toxicity, and exposure to TCDD clearly impairs T cell-dependent immune responses, which contribute to promote inflammatory autoimmune diseases[2-4]. Aryl hydrocarbon receptor (AhR) has been considered as one of the key molecules mediating the aforementioned toxic effects. Moreover, increasing experimental and epidemiological studies have shown that the AhR-dependent signaling pathway plays an important role in normal immune functions, particularly in the realm of CD4$^+$ helper T (Th) cell mediated inflammation[5, 6]. Up to now it is not clear whether the TCDD mediated effects are indirect results from the emerging modulatory function of AhR in the immune system, or attributable to direct TCDD exposure induced toxicity in target cells. Since CD4$^+$Th cells play an integral role in adaptive immune responses, and the differentiation of Th cell subsets is tightly regulated to evoke appropriate immune responses, any disturbance of this process might result in the induction of undesirable inflammatory autoimmune diseases. Therefore, we aim to investigate the involvement of potential modulatory effects of dioxin in its disturbance of CD4$^+$Th cell differentiation using two different culturing conditions *in vitro*: isolated CD4$^+$Th culture and whole mixed spleen lymphocyte culture (SLC).

Materials and methods

Animals. Eight to ten-week-old male C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China). All animal experiments were carried out in accordance with the approved guidelines by the Institutional Animal Care and Use Committees of the Institute of Psychology, Chinese Academy of science.

Antibodies and Other Reagents. FITC-labeled anti-CD4, PE-labeled anti INF-γ, PE-Cy7-labeled anti-IL4, Alexa 647 labeled anti-IL17, Hrizon V450 labeled anti-Foxp3 antibodies, staining buffer and transcription factor buffer set were purchased from BD Bioscience (San Diego, CA). Carboxyfluoresceindiacetatesuccinimidyl ester (CFSE) was purchased from eBioscience (San Diego, CA). Concanavalin A (Con A) was purchased from Sigma-Aldrich (St. Louis, MO). A luminescent cell viability assay was used to evaluate the cytotoxicity, and the assay kit was purchased from Promega (Madison, WI). TCDD was purchased from Wellington Laboratories Inc. (Ontario, Canada).

Preparation of spleen lymphocytes and target cell isolation. Dissection of C57BL/6 mouse spleen was carried

out in sterile conditions. We disrupted the spleen in PBS with 2% FBS and 1mM EDTA. The cell suspension was passed through a 70 μm nylon cell strainer filter into a 50 mL conical tube, and rinsed with up to 20 mL of medium. Pass-through was centrifuged (1500rpm, 10min) and re-suspended at 1-1.5x10^8 cells/mL in recommended medium. The obtained spleen single cell suspension was subjected to $CD4^+$ cell Pre-Enrichment according to the manufacturer's instruction (STEMCELL Technologies Inc, Vancouver, Canada).

In vitro cell expansion and culture. The isolated $CD4^+$Th cells were seeded at 2×10^6 cell/1.4 mL/well in 12-well-plates in a modified RPMI1640 medium with 10% FBS, 100U/mL penicillin and streptomycin, 2mM glutamine, 50μM β-mercaptoethanol, 10mM HEPES and 0.5mM sodium pyruvate. Anti-mouse CD3 and CD28 antibody-coated 4μm polystyrene dynabeads (Invitrogen, Carlsbad, CA) at 1:1 ratio, and 2000U/mL or 30U/mL recombinant mouse IL2 (PeproTech, Rocky Hill, NJ) were added for $CD4^+$Th cell culture *in vitro*. To monitor and evaluate the proliferation of target cells, CFSE was used according to the manufacturer's instruction.

Cytokines Staining and Flow Cytometry. Four days after Con A (4 μg/mL) and TCDD (T1: 20nM; T2: 200 nM) co-treatment, cells were collected and washed once with PBS and once staining buffer once. Cell suspension containing 1 million cells per 150 μL was prepared in staining buffer, and stained by cell surface antibodies and finally by intracellular antibodies. . Treatments of TCDD solvent, dimethyl sulfoxide (D) were served as controls.

Results and discussion

When spleen $CD4^+$Th cell were magnetically isolated from C57BL/6 and cultured for 5 days, no significant changes in cell viability was detected during the culturing period. Cells were exposed to different concentrations of TCDD (4nM to 200 nM) together with Con A for 4 days, then cell number was counted after trypan blue staining. Results showed that significant suppression of cell proliferation was observed but without obvious cell toxicity. The suppressive effect of TCDD on $CD4^+$Th cells was further investigated in terms of the ratio of differentiated subsets induced by Con A. Multicolor flow cytometry was employed to evaluated the differentiation. Results showed that after a 4-day-exposure to TCDD *in vitro*, moderate decreases in the distributions of Th1, Th2, Th17 and Treg cell types were observed. The suppressive effect of TCDD was obvious at 20 nM, while treatment at 200 nM didn't further decreased the distribution of the subtypes (Table 1).

To address whether immune-modulatory effects induced by TCDD were involved in its interference with the differentiation of $CD4^+$Th cells, we further investigated the effects of TCDD in SLC condition. Unlike the effect found in isolated spleen $CD4^+$Th cell cultures, the immune suppression on Th2 and Th17 cells appeared only after exposure of TCDD at 200 nM without effect at lower concentration, but the differentiation of Th1 cells was further suppressed by exposure to 200 nM TCDD (Table 1). The suppression of Treg cells by TCDD did not change in the two culture conditions, suggesting that interference of Treg differentiation by dioxin mostly results from direct actions (Table 1). In contrast, the reduced sensitivity of Th2 and Th17 to TCDD might be attributed to the potential modulatory effect of dioxin on spleen lymphocytes other than $CD4^+$Th cells, which needs further investigations. Th2 cells were remarkably increased under SLC condition, while Th17 subtypes were decreased. (Table 1). This interesting finding might be able to provide some hints for elucidating the indirect mechanisms for TCDD induced immune suppression on $CD4^+$Th cells.

Table 1 Distinct suppression of TCDD on subtype distribution of $CD4^+$Th in two different culturing conditions

	$CD4^+$Th			SLC		
	D	T1	T2	D	T1	T2
Th1	19.6%	14.7%	14.3%	13.9%	10.3%	6.7%
Th2	19.1%	13.5%	15.3%	55.9%	56.8%	50.7%
Th17	25.9%	21.2%	20.4%	2.5%	2.5%	1.7%
Treg	36.9%	29.9%	29%	25.5%	20%	19.3%

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