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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin modulates the expression of cKrox and Runx3, transcription regulatory factors controlling the lineage commitment of $CD4^+CD8^+$ into CD4 and CD8 thymocytes, respectively

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was reported to skew the lineage commitment of thymocytes toward $CD4^-CD8^+$ T (CD8 T) cells. However, the underlying mechanisms are not known. In the present study, we first demonstrated that the expression of transcription regulatory factors such as cKrox and Runx3, which have been shown to be intimately associated with the commitment of $CD4^+CD8^+$ double-positive (DP) to CD4 or CD8 single-positive (SP) thymocyes, was down-regulated by TCDD in CD4 SP thymocytes, but up-regulated in DP, $CD4^+CD8^+$ double-negative (DN), and CD8 SP thymocytes. Then, we found that TCDD inhibited the differentiation of DPK cells, an immature $CD4^+CD8^+$ lymphoma cell line, into $CD4^+CD8^-$ T cells, as well as the expression of cKrox and Runx3 upon antigen stimulation. Co-treatment with the AhR antagonist α-naphthoflavone did not completely block the inhibitory action of TCDD on DPK differentiation and the expression of cKrox and Runx3 in DPK cells, suggesting that the immunomodulatory abilities of TCDD are produced, at least in part, independently of the AhR pathway in DPK cells. Our findings could help in understanding the regulatory mechanisms of TCDD on thymocyte development, in particular on the skewed differentiation of DP into CD8 SP thymocytes.

1. Introduction

Developing αβ T cells progress through three major stages in the thymus, defined by differential expression of the CD4 and CD8 co-receptor molecules; $CD4^-CD8^-$ (double-negative; DN), $CD4^+CD8^+$ (double-positive; DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ (single-positive; SP). The development of $CD4^+CD8^+$ to $CD4^+$ or $CD8^+$ T cells, termed positive selection, which consists of a number of temporally and mechanically distinct cellular changes, including rescue from apoptosis, cell differentiation, and commitment to the CD4 or CD8 T cell lineage, depends on productive rearrangement of both α- and β-subunits of the T cell receptor (TCR) and TCR-mediated recognition of major histocompatibility complex (MHC) molecules expressed by thymic stroma.

Thymocytes expressing the DP phenotype undergo continuous TCRα rearrangement to yield a $TCR\alpha\beta^+$ population that is selected for additional maturation depending on the binding affinity of individual TCRαβ heterodimers for self-MHC molecules (Wang et al., 1998). The majority of DP cells die because of not properly engaging self-MHC/peptide complexes, and thymocytes which bind with high affinity to MHC/peptide complexes are deleted by negative selection (von Boehmer et al., 1989). Thymocytes which bear TCRs of intermediate affinity for self-MHC/peptide complexes survive and further differentiate to SP cells by a process called positive selection (Jameson et al., 1995).

Mature T cells expressing CD4 have TCR specificity towards MHC class II molecules whereas CD8-expressing T cells have TCR reactive with MHC class I molecules. Alternative instructive and stochastic models have been proposed to explain this marked correlation (Germain, 2002). According to the instructive model, receptor/co-receptor matching is imparted during the selection process: the class of MHC molecule engaged by the TCR is sensed by the cell undergoing selection, and this information directs the choice of proceeding along the CD4 or CD8 pathway. Recently, a general 'strength of signal' model evolved from the instructive model, proposing that the strength and/or duration of TCR signaling determines lineage choice, with strong/long signals promoting the CD4 T cell lineage and weaker/shorter signals favoring the CD8 T cell lineage (Germain, 2002; Liu and Bosselut, 2004). The stochastic model, on the other hand, proposes that differentiation continues along one or the other pathway independently of the cell's preference for

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, arylhydrocarbon receptor; DP, double positive; SP, single positive; TCR, T cell receptor; MHC, major histocompatibility complex.

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MHC class; whether the proper choice was made is secondarily confirmed as one of the co-receptors is down-regulated, and only the cells which have made the right decision survive and finalize maturation (Robey et al., 1994). Current thinking favors the strength of signal model.

Signaling pathways implicated in positive selection and lineage commitment have been studied. MAP kinase and calcineurin signaling pathways have been shown to be required for positive selection (Swan et al., 1995; Pages et al., 1999; Bueno et al., 2002), and the extent or timing of activation of MAP kinase and calcineurin signaling pathways could determine the CD4/CD8 lineage decision. CD4 lineage commitment was shown to be sensitive to levels of MAPK inhibitors that had no effect on CD8 maturation (Bommhardt et al., 1999). In addition, CD4 differentiation required longer duration of MAPK signals than required for CD8 lineage commitment (Wilkinson and Kaye, 2001). Similarly, the calcineurin inhibitor FK506 at 1 nM inhibited the development of thymocytes to either lineage, but 0.3 nM FK506 significantly switched the development from the CD4 lineage to the CD8 lineage (Adachi and Iwata, 2002).

Considerable effort has been devoted to identifying transcription factors essential for CD4/CD8 lineage commitment. GATA-3, a zinc finger transcriptional activator, seems to promote maturation or survival of thymocytes that have already chosen the CD4 fate (Hernandez-Hoyos et al., 2003). TOX, a HMG box protein, was shown to be associated with the DP to SP transition (Wilkinson et al., 2002; Aliahmad and Kaye, 2008). However, the role of TOX in the lineage commitment is controversial. In one study using transgenic mice over-expressing TOX, TOX directed the DP to CD8 SP transition (Wilkinson et al., 2002), whereas in other study using TOX-deficient mice, it appeared that TOX is necessary for the DP to CD4 SP transition (Aliahmad and Kaye, 2008). In contrast to GATA-3 and TOX, there is accumulating evidence that Runx3 and cKrox are intimately associated with the lineage commitment. Runx3, a member of a family of transcriptional regulators that contain a conserved 128 amino acid DNA-binding Runt domain, is expressed higher in CD8 thymocytes than in CD4 thymocytes (Taniuchi et al., 2002) and promotes the expression of the CD8 gene while silencing the CD4 gene in developing $CD8^+$ thymocytes (Sato et al., 2005). cKrox (also called Th-POK or Zbtb7b), a zinc finger transcriptional regulator, was shown to direct the DP to CD4 SP transition (He et al., 2005; Sun et al., 2005) and to directly or indirectly suppress the activity of Runx3 (Wildt et al., 2007).

Halogenated aromatic hydrocarbons (HAHs) are a class of structurally related compounds that are widespread environmental contaminants. Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) is the most potent congener of the HAHs, causing a broad spectrum of toxic effects including immune, reproductive, and developmental toxicity (Huff et al., 1994). In mice, TCDD has been shown to reduce thymocyte numbers and modulate thymocyte differentiation, skewing the lineage commitment of thymocytes into $CD4^-CD8^+$ SP thymocytes (Poland and Knutson, 1982; Tsukumo et al., 2002; Temchura et al., 2005). Thymus atrophy and suppressed immune functions induced by TCDD was shown to be mediated by the activation of aryl hydrocarbon receptor (AhR) (Staples et al., 1998; Vorderstrasse et al., 2001). However, despite intensive study, the mechanisms underlying the impaired immune responses induced by TCDD remain poorly understood.

To have insights into the mechanisms underlying the skewed differentiation of DP thymocytes into $CD8^+$ SP thymocytes by TCDD, we first examined the expression of cKrox and Runx3, transcription regulatory factors which have been shown to be intimately associated with the commitment of $CD4^+CD8^+$ DP to CD4 or CD8 SP thymocyes, in four subpopulations of thymocytes from TCDD-treated mice. Then, we cultured $CD4^+CD8^+$ DPK lymphoma cells, which can differentiate into $CD4^+CD8^-$ T cells upon TCR stimulation in vitro (Kaye and Ellenberger, 1992; Shao et al., 1997; Oda et al., 2007), in the presence or absence of TCDD to investigate the effects of TCDD on the differentiation of DPK.

2. Materials and methods

2.1. Mice and TCDD administration

BALB/c mice, 6–12 weeks of age, were purchased from the Korean Institute for Chemistry (Taejon, Chungnam, Korea). TCDD in nonane was diluted with corn oil and injected intraperitoneally (i.p.) (30 μg/kg body weight).

2.2. Cells

DPK cells, a $CD4^+CD8^+$ lymphoma cell line (Kaye and Ellenberger, 1992), were maintained in EHAA medium (In Vitrogen, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and antibiotics and mycostatin. DCEK-ICAM cells, a fibroblast cell line which expresses the murine class II MHC genes E^k_α and E^k_β specific for pigeon cytochrome *c* peptide 88–104 and the murine ICAM-1 gene (Paul Sung et al., 1992), were maintained in RPMI 1640 medium (In Vitrogen, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics and antimycotics.

2.3. Chemicals and reagents

α-Naphtoflavone and benzo(*a*)pyrene, and TCDD were purchased from Sigma–Aldrich (USA) and from Cambridge Isotope (USA), respectively.

2.4. DPK cell differentiation

5×10^6 DECK-ICAM cells are treated with mitomycin C (50 μg/ml, 30 min, 37 °C), washed with HBSS with 5% FBS, and plated on 10 cm tissue culture plates. After 24 h, DECK-ICAM cells were treated with 3 μM pigeon cytochrome *c* peptide KAERADLIAYLKQATAK for 1 h and cocultured with 3×10^6 DPK cells for 2–4 days.

2.5. In vitro treatment of TCDD and α-naphthoflavone

Chemicals were commonly used as stocked solution in DMSO. The final concentration of the solvent in culture medium did not exceed 0.1% (v/v), and DMSO control cultures were treated with 0.1% DMSO. TCDD was used at concentrations of 1–100 nM, which has been shown to be active in previous studies (Park et al., 2003; Cho et al., 2006).

2.6. RNA preparation, reverse transcription-polymerase chain reaction (RT-PCR), and real-time PCR

Total cellular RNA was extracted from cells using the RNAzol method (Tel-Test, Inc., USA). For PCR analysis, RNA was used after contaminating DNA was completely removed by DNase I treatment. RT-PCR analysis was performed using pairs of oligonucleotide primers. The PCR products were confirmed to correspond to their original sequence by DNA sequencing. PCR products were comparatively quantitated by GS-700 densitometer (Bio-Rad, USA). Gene specific primers, number of cycles of amplification, annealing temperature, and expected size of PCR product are listed in Table 1. Real-time PCR was performed to confirm the RT-PCR results. Power SYBR green PCR Master Mix and Real-time PCR system (7300, Applied Biosystems, USA) were used.

2.7. Flow cytometry

Cells were kept on ice or at 4 °C at all time. Immunofluorescence staining was carried out in 96-well, round-bottomed cell culture plates. The cells were centrifuged and resuspended in 10 μl of primary antibody diluted with PBS at a previously determined optimal concentration. The following antibodies were used: CD4, clone GK1.5 (Pharmingen, USA); CD8α, clone 53–6.7 (BioLegend, USA). After 15 min of incubation, the cells were washed with washing buffer (PBS/0.5% BSA/0.09% sodium azide) three times and fixed with 0.9% buffered formalin. Cells were analyzed within 1 week on a FACSCalibur flow cytometer (Becton Dickinson, USA).

2.8. Magnetic cell sorting

$CD4^+$ and $CD8^+$ SP thymocytes were isolated from total thymocyte suspensions using MACS Anti-FITC Mutisort kit and CD8a (Ly-2) micrbeads (Miltenyi Biotech, Germany), and FITC-anti-CD4 Ab (clone GK1.5, Pharmingen, USA).

2.9. Statistical analysis

Data were analyzed with the paired Student's *t* test. The level of significance was $p < 0.05$.