Skewed emigration of CD4/CD8 thymocyte subpopulations from murine fetal thymi exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin: *In vitro* studies on distribution and CD44 expression

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is an immunosuppressive chemical[1]. Exposure of laboratory animals leads to reduced size and cellularity of lymphoid organs such as thymus, spleen and lymph nodes. Apparently TCDD interferes with the differentiation of cells of the lymphoid lineage. In the thymus cytotoxic T-cells and helper T-cells are generated. Each day about 10E6 newly matured and selected T-cells leave the thymus to circulate in the periphery. T-cells there either meet their respective antigen and become effector cells or die in due course. The number of T-cells in the periphery is subject to homeostatic processes. In the adult and probably also old animal the overall number of T-cells remains the same, although the thymus physiologically atrophies[2]. We have studied in depth the effects of TCDD on thymocyte proliferation and differentiation. We showed that TCDD causes a reduced proliferation rate of the earliest immigrated thymocytes and a skewed differentiation of certain thymocyte subpopulations, in particular the CD4-CD8+ thymocytes. These cells were shown to be phenotypically and functionally equivalent to cytotoxic T-cells, whose main function is to protect the body against viral infections and tumor cells. TCDD-exposure of laboratory animals results in a decreased ability to fight such infections. Moreover, we could show that TCDD acts on the thymocytes indirectly, via the surrounding thymus epithelial cells, probably by inducing aberrant cytokine signals[3]. However, although it has often been inherently assumed that thymus atrophy (including all the differentiation events affected by TCDD) and the general immunosuppression caused by TCDD are causally related, up to now no link between events in the thymus and the immune system present in the body has been experimentally established. We therefore asked whether TCDD interferes with emigration of cells from the thymus, i.e. (a) how many and (b) which thymocytes/T-cells emigrate. We used fetal thymus organ cultures of murine thymi as an in-vitro model of thymocyte emigration. Cells were classified according to their maturation status by staining with CD4 and CD8 and additionally by their expression of CD44 and other relevant markers.

Methods: Mice of the genetically dioxin-sensitive strain C57Bl/6 were used in this study. Thymi from fetuses of day 15 of gestation were excised and cultivated as complete organs on nitrocellulose filters. The filters were placed on top of culture medium so that the thymi were lying on the liquid-air interphase. Under these conditions thymocytes proliferate and differentiate ex vivo parallel to the in utero development. Thymi were cultured either in the presence or absence of 10mM TCDD. After culture the thymi were flushed three times with 2 x 200µl of phosphate buffered saline to collect cells ("emigrants") that had left the thymi. Afterwards the intact thymi were picked from the nitrocellulose filter and gently homogenized to release the thymocytes from within. Both cell populations (emigrants and thymocytes) were counted and stained with fluorescence-labelled antibodies against specific surface markers and analysed flow cytometrically on a FACScan flow cytometer.

Results: Fetal thymi were exposed to TCDD for up to six days in organ culture. Thymocytes were then triple stained with CD4, CD8, and CD44 or the two different types of the T-cell receptor (TCR), the alpha-beta TCR or gamma-delta TCR. Tables 1 and 2 show the results of a representative experiment of day six of organ cultures. As we have reported before, exposure to TCDD skewes the distribution of mature thymocytes/T-cells in the thymus to the prospective cytotoxic T-cell, i.e. the CD4-CD8+ single positive cells. In the experiment shown 25% of CD4-CD8+ cells are found compared to a normal 4% in the absence of TCDD. Correspondingly the frequency of CD4+CD8+ cells is decreased. We collected from the surface of the filters on which the thymi were placed emigrated thymocytes. About 1/10th of the cells counted within one lobe are found outside. For the unexposed thymi the frequency pattern of thymocytes outside reflects the pattern from the inside (compare the control values in Table 1). In contrast, emigrated cell from TCDD-exposed thymi have a quite different distribution. The CD4+CD8+ double-positive cells are preferentially retained in the thymus, whereas the CD4-CD8- double negative cells preferentially leave the thymus. CD4-CD8- thymocytes represent the most immature precursors from which the other thymocytes are generated by the maturation processes. More of the mature, prospective cytotoxic T-cells emigrate from the thymus as well. Also in absolute terms, more double-negative cells are found outside.

CD44 is a surface molecule that is involved in migration of thymocytes/T-cells via epithelial tissue, for instance to home into lymphoid organs[4]. We therefore analysed the distribution of CD44 on the thymocyte emigrants. Interestingly, more cells of both immature maturation stages (CD4-CD8- and CD4+CD8+) bear CD44 on their surface, as well in the inner thymocytes as in the emigrants. In the emigrants up to 85% of the double-negative cells are CD44 positive. Also the other thymocyte subpopulations have an increased part of CD44 positive cells. The CD4-CD8-CD44+ emigrants do not belong to a mature subset of thymocytes that express the gamma-delta T-cell receptor, as we confirmed by staining. The preferential emigration of double-negative cells begins as early as day 1 of the culture (not shown).

TABLE 1

Frequency-kinetics of different maturation stages of thymocytes and thymic emigrants in the presence or absence of 10 nM TCDD in fetal thymus organ cultures

	Inner thymocytes		*Emigrants*	
	ccontrol	TCDD	control	TCDD
CD4-CD8-[b)]	3	19	8	**58**
CD4+CD8+	77	39	69	**17**
CD4+CD8-	15	17	20	11
CD4-CD8+	4	25	4	**14**

a) Fetal thymi were organ cultured for six days as described in Methods and the thymocytes released by gentle homogenization (inner thymocytes). Cells which had emigrated from the thymus and could be found outside on the nitrocellulose filter on which the thymi had been cultured were collected and stained separately as „emigrants".

b) CD4 and CD8 are surface molecules on thymocytes. Their differential expression is correlated to the maturation stage of the thymocyte. The CD4-CD8- are the most immature thymocytes, the CD4+CD8+ cells express already the T-cell receptor and are selected for the immune system. They make up the majority of thymocytes. CD4+CD8- or CD4-CD8+ cells are the mature helper or killer T cells, respectively, ready to emigrate from the thymus.

TABLE 2
Frequency of CD44 bearing cells within different thymocyte maturation stages upon TCDD exposure

		Inner thymocytes[a]				*Emigrants*[a]			
		CD44 negative		CD44 med/hi		CD44 negative		CD44 med/hi	
	days[b]	control	TCDD	control	TCDD	control	TCDD	control	TCDD
	0	75 %[c]		26 %		75 %		26 %	
CD4-CD8-	6	53	53	33	47	39	16	61	**85**
CD4+CD8+	6	90	83	10	17	94	65	7	**35**
CD4+CD8-	6	90	85	10	15	94	65	6	**35**
CD4-CD8+	6	79	86	21	14	62	53	31	39

a) fetal thymi were organ cultured for several days as described in Methods and the thymocytes released by gentle homogenization (inner thymocytes). Cells which had emigrated from the thymus and could be found outside on the nitrocellulose filter on which the thymi had been cultured were collected and stained separately as „emigrants".
b) days of organ culture. Counting starts with 0 on day 15 of gestation when thymi were taken from the fetuses. Thymi were organ cultured in the presence of 10 nM TCDD/0.1% dioxane, or with this solvent alone (control).
c) frequency of cells bearing CD44 on their surface. Cell were triple stained with anti-CD4, anti-CD8, and anti-CD44 antibodies and 10000 cells were flow-cytometrically analysed.

Conclusion: It is is important to experimentally assess the connection between events in the thymus that are affected by TCDD exposure even at very low doses and the general immunosuppression ascribed to this chemical. In a first study we have phenotyped emigrating thymocytes that matured in the presence or absence of TCDD. We have found that TCDD-exposure results in a dramatic shift of emigrating cells. In particular the very immature cells preferentially leave the thymus. This may add to the reduction of the number of thymocytes by TCDD, which was shown to be caused by a reduction of the proliferation rate of the precursors (CD4-CD8-) by about 30%. The reduction of cell number in turn causes the thymus atrophy, which has been known for a long time to be the hallmark of TCDD-exposure of laboratory animals. The most interesting finding of this study, however, is the high frequency of CD44 expressing cells in the different maturation stages. It remains to be shown whether this is caused by a direct induction of the CD44 gene via the activated Ah-receptor, or whether CD44 upregulation is yet another one of the multiple indirect changes induced by TCDD in the thymus. It is not known whether the promoter of CD44 contains dioxin-responsive elements. CD44 is a molecule that directs T-cells to enter lymphoid organs by allowing them to cross the epithelial barriers. Different isoforms of CD44 may be involved in entering specific types of tissues. Therefore, it will be very interesting to analyse whether T-cells generated in TCDD-exposed thymi have a changed behaviour of circulating in the blood stream, lymph and lymphoid organs. We are currently investigating this question by following the course of recent thymic emigrants after in vivo labelling with fluorescent markers.

Acknowledgement
This work is supported through SFB503, project C5 "Endogenous mediators of exogenous noxes" at the Heinrich-Heine-University of Düsseldorf. We are grateful for the expert and diligent assistance of Swantje Steinwachs and Martina Welzel.

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