2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to development of mature CD8 cells in MHC-I deficient mice

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Abstract
2,3,7,8-tetrachlorodibenzo-*p*-dioxin skews the differentiation of thymocytes towards the future cytotoxic $CD4^-CD8^+$ T-cells. The selection of $CD4^+CD8^+$ thymocytes into the $CD4^-CD8^+$ compartment normally requires the interaction of the T-cell receptor on the surface of the thymocytes with the MHC-class I molecules on the stromal cells of the thymus. Thus, mice lacking MHC-class I molecules due to gene-targeted mutation do not produce cytotoxic T-cells any longer. We report here, that TCDD causes the generation of large numbers of CD8-positive cells in such mice, suggesting interference with the pathway that causes the switching off of the CD4 molecule upon T-cell receptor/MHC-I interaction.

Introduction
Thymocytes differentiate in a well described mode from the earliest intrathymic precursors to naive T-cells, which emigrate from the thymus. At the stage of $CD4^+CD8^+\alpha\beta TCR^+$ expression the thymocytes are selected on the basis of their ability to recognize foreign peptides in the context of self-major histocompatibility complex (MHC) class I or class II molecules. Thymocytes with a $\alpha\beta$TCR specific for MHC-class I of class II downregulate the CD4 or CD8 molecule, respectively. The normal ratio of $CD4^+CD8^-$ cells *versus* $CD4^-CD8^+$ cells in the thymus is about two. Upon exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) this differentiation is skewed towards $CD4^-CD8^+$ thymocytes, up to 40% $CD4^-CD8^+$ cells can be generated in fetal thymus organ cultures (FTOC). These cells express normal maturation markers and are functionally cytotoxic in the ^{51}Cr-release assay[1]. However, positive selection of thymocytes, i.e. the survival of cells after appropriate interaction with self-MHC-molecules is disturbed by TCDD[2], more cells audition for and pass positive selection. We therefore test here in mice lacking MHC-class I molecules, whether the $CD4^-CD8^+$ cells – that are so abundantly generated in the presence of TCDD – are restricted to MHC-class I, i.e. need the interaction with this molecule for their generation/survival.

Experimental Methods
Mice homozygous for the disrupted β_2 microglobulin gene were purchased from Genpharm Int. (Mountain View, USA). These mice contain no functional MHC-class I molecules. For controls female C57BL/6 mice were used. All mice were of the $H\text{-}2^h$ haplotype. Fetal thymus organ cultures from timed pregnancies were set up for up to seven days in serum-free medium, with no external source of $\beta_2$microglobulin. TCDD dissolved in 1,4-dioxane or solvent alone was added at a concentration of 10 nM.

For *in vivo* experiments, pregnant mice were injected on gestation day 15 with 50 µg /kg body weight 2,3,7,8-TCDD, and the thymi of their newborn pups were analysed.
Thymocytes were stained with fluorochrome-conjugated cell surface antibodies and analysed in a FACScan flow cytometer.

Results and Discussion

As expected, whereas in the thymi of C57BL/6 mice about 5-10% $CD4^-CD8^+$ thymocytes were detectable, virtually no cells of this subpopulation were generated in the $\beta_2m^{-/-}$ thymi (Fig. 1). However, upon addition of TCDD to the medium, we detected in the thymi of both strains $CD4^-CD8^+$ single positive cells at similar, high frequencies.
Day 7 of culture equals the day of parturition and the CD4/CD8 subpopulation pattern of FTOC controls corresponds to that of newborn mice. The presence of TCDD in the medium led to generation of $CD4^-CD8^+$ cells, irrespective of the presence or absence of MHC-class I molecules. Concomitantly, the frequency of $CD4^+CD8^+$ cells was reduced, as had been reported numerous times before. Note that the frequency of $CD4^-CD8^-$ cells – the very immature subset – remained high in the $\beta_2m^{-/-}$ FTOC. This is most likely due to the medium, which lacks fetal calf serum and may not optimally support the organ cultures in the presence of the "stress factor" TCDD. To further investigate the CD8 cells which are generated despite the lack of MHC-class I molecules, we tested the maturation markers αβTCR, CD3 and HSA. The results are summarized in Fig. 2.
Mature thymocytes ready to emigrate into the periphery as naive T-cells bear the αβTCR and CD3. In contrast, HSA is a marker of immature thymocytes, and $CD4^-CD8^+HSA^+$ are an early intermediate maturation stage. Compared to the C57BL/6 control mice fewer CD8 cells bear the maturation markers αβTCR and CD3 in $\beta_2$microglobulin deficient mice upon TCDD exposure. However, 20 - 30 % of these $CD8^+$ single positive cells can be considered phenotypically mature cells according to these markers. High expression of the αβTCR on thymocytes is thought to be

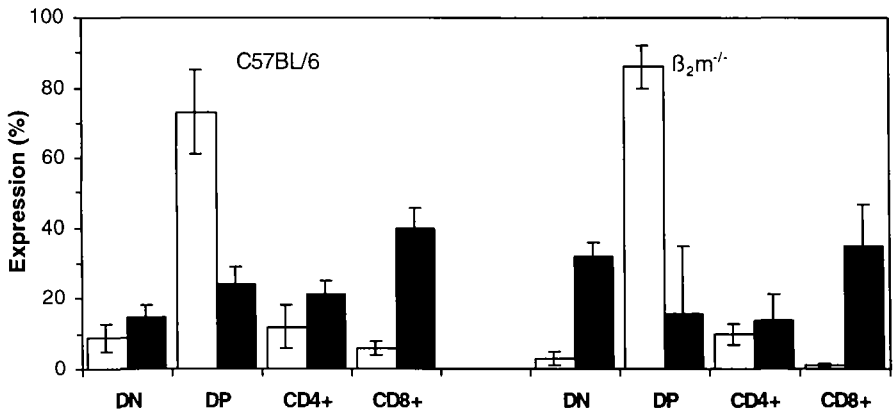

Figure 1. Fetal thymus organ cultures from C57BL/6 mice or MHC-class I deficient ($\beta_2m^{-/-}$) mice were set up for 7 days either untreated (□) or treated with 10 nM TCDD (■). Thymocytes were stained and the frequency of the four thymocyte subsets $CD4^-CD8^-$ (double negative, DN), $CD4^+CD8^+$ (double positive, DP), $CD4^+CD8^-$ and $CD4^-CD8^+$ was determined.

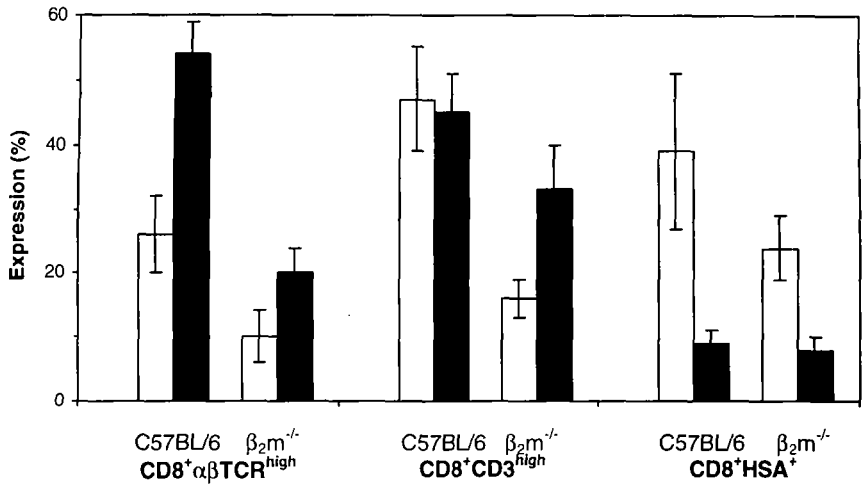

Figure 2. Thymocytes generated in fetal thymus organ cultures were stained for CD4, CD8 and either αβTCR, CD3 or HSA differentiation markers. The frequency of $CD4^-CD8^+$ cells bearing these marker are shown for thymocytes derived from C57BL/6 and $\beta_2m^{-/-}$ fetal thymi, either untreated (□) or treated with 10 nM TCDD (■).

a marker of successful positive selection and maturation. Expression of αβTCR and CD3 was high, normally a foot-print of successful positive selection and maturation. Also in absolute cells/thymus lobe, CD8 cells were increased in fetal thymi of $\beta_2$microglobulin deficient mice treated with TCDD (data not shown). Moreover, staining with HSA confirmed the shift towards mature CD8 thymocytes in TCDD-exposed organ cultures. In the C57BL/6 fetal thymi as well as in the $\beta_2m^{-/-}$ thymi the frequency of HSA^{high}-expressing cells decreased. To confirm our findings in an *in vivo* situation, we analyzed the thymi of newborn $\beta_2m^{-/-}$ pups, which had been exposed for seven days *in utero* to TCDD. As shown in Fig. 3, about 7 % $CD4^-CD8^+$ cells develop in MHC-I-deficient mice, whereas – as expected – no $CD4^-CD8^+$ cells are found in the control C57BL/6 mice. In accordance with the findings in the FTOCs, part of the newly generated CD8-single positive cells of neo-nates exposed to TCDD *in utero* are mature cells as judged by the expression of αβTCR, CD3 and HSA (data not showon).

The pharmacokinetics of TCDD injected *i.p.* is not clear, thus the actual dose to which the thymi of the embryos were exposed is unknown. However, the dose used *in vivo* is effective, although no direct comparison is possible to the 10 nM used in the fetal thymus organ cultures. Both doses led to a comparable reduction in thymocyte numbers, in the fetal thymus organ cultures and in the thymi of newborn pups. The absolute number of $\alpha\beta TCR^{high}CD8^+CD4^-$ cells increased in both cases up to 15 times (data not shown).

Thus, also *in vivo* TCDD leads to the undogmatic generation of phenotypically mature CD8 cells. It is not likely that TCDD simply down-modulates CD4 on $CD4^+CD8^+$ thymocytes, independent of MHC-class I contact. Transcriptional modulation by TCDD via the liganded Ah-receptor has been described for a battery of genes. However, the CD8 cells generated are phenotypically different from $CD4^+CD8^+$, in that they express the αβTCR and CD3 at high levels, signs of additional maturation events. Moreover, the CD8 cells generated in C57BL/6 mice can effectively lyse target cells, and can thus be considered functionally mature.

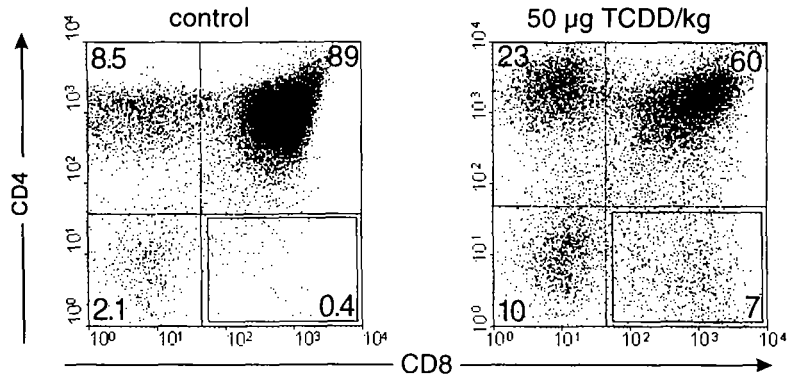

Figure 3. Flow cytometric analysis of CD4 versus CD8 expression in the thymocytes of either C57BL/6 of β_2m deficient newborn mice. Pregnant dams had been injected on gestation day 15 with corn oil or 50 µg TCDD/kg body weight.

Some data from the literature indicated that $CD4^-CD8^+$ lymphocytes from $\beta_2m^{-/-}$ mice could differentiate independently of MHC-I recognition. Thus, few $CD4^-CD8^+$ thymocytes in $\beta_2m^{-/-}$ mice might by-pass positive selection and differentiate without selection[3], and TCDD would just promote the outgrowth of these cells. $\beta_2$microglobulin forms the MHC-class I molecule together with another protein, the α-chain. At very low frequency, the α-chain can form aberrant antigen-presenting MHC-molecules without $\beta_2$microglobulin. It might be possible that $CD4^-CD8^+$ thymocytes are MHC-I restricted on such molecules. However, it is hard to explain how the high frequency of phenotypically mature $CD4^-CD8^+$ cells in the TCDD-exposed FTOCs of $\beta_2m^{-/-}$mice is generated. It appears unlikely that few (more or less) functional MHC-class I molecules are sufficient to select thymocytes into the $CD4^-CD8^+$ pool at a high frequency and absolute number equal to that of non-mutant mice in the presence of TCDD.

A more likely explanation of the generation of $CD4^-CD8^+$ cells in $\beta_2$microglobulin-deficient mice is their potential class II restriction. $CD4^-CD8^+$ T-cells recognizing class II MHC molecules have been described by several groups. The recognition of MHC-II by $CD4^-CD8^+$ T cell clones was shown to be αβTCR-mediated, but independent of the CD8 coreceptor. TCDD would appear to promote this normally rare process drastically, opening an alternative pathway of development which is masked in wildtype thymi. Thus, the TCDD-induced CD8-cells can be used to study further the mechanism of commitment to the MHC-I/CD8 restriction.

Whether or not our finding bears upon the systemic immunosuppression caused by TCDD-exposure is currently investigated.

References

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