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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) 1 on adult  
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1 **Direct and indirect impact of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD)**  
2 **on adult mouse Leydig cells: an in vitro study.**

3

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7

8 Short title: Impact of TCDD on cultured Leydig cells

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19

20

21 **Abstract:**

22 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related substances are ubiquitous  
23 environmental pollutants that exert adverse effects on reproductive processes. In testis,  
24 Leydig cells which produce testosterone are under hormonal and local control exerted by  
25 cytokines including TNF $\alpha$ . Using mouse Leydig primary cell cultures as a model, we studied  
26 the effects of TCDD on the steroidogenic outcome of Leydig cells and the gene expression  
27 levels of Ccl5 and Cxcl4, previously shown to be target genes of TCDD in testis. We found  
28 that TCDD did not alter the steroidogenic outcome of Leydig cells but that it up-regulated  
29 Cxcl4 gene expression levels. TCDD also impacted Ccl5 gene expression when cells had been  
30 co-treated with TNF $\alpha$ . TCDD action probably initiated with binding to the aryl hydrocarbon  
31 receptor (AhR) present on Leydig cells. TCDD regulated the gene expression levels of AhR  
32 (transient down-regulation) and its repressor AhRR and Cyp1b1 (up-regulation). The trophic  
33 human chorionic gonadotropin (hCG) hormone did not impact AhR, its repressor AhRR or  
34 Cyp1b1 but it opposed the TCDD-enhanced AhRR mRNA levels. Conversely, TNF $\alpha$   
35 stimulated AhR gene expression levels. Collectively, it is suggested that the impact of TCDD  
36 on expression of target genes in Leydig cells may operate under the complex network of  
37 hormones and cytokines.

38

39 Key words: Dioxin; Leydig cell; in vitro; AhR; chemokine

40

## 41 **1. Introduction**

42 Polycyclic aromatic hydrocarbons (PAHs), as well as halogenated aromatic  
43 hydrocarbons (HAHs) such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) and related  
44 compounds, are highly persistent environmental pollutants. Dioxins are by-products from  
45 incineration processes and pesticide production, and thus they are not intentionally produced.  
46 They are considered endocrine disruptors (Diamanti-Kandarakis *et al.* 2009; Hotchkiss *et al.*  
47 2008; Lundqvist *et al.* 2006), i.e. chemical substances that interfere with the endocrine  
48 systems of humans and wildlife. As such, they are of high concern for human health because  
49 endocrine disruptors are suspected to be responsible for apparent changes seen over recent  
50 decades, including congenital malformations, cancer and declining sperm counts (Skakkebaek  
51 *et al.* 2001; Toppari *et al.* 1996). Dioxins are not genotoxic but cause a broad spectrum of  
52 adverse effects including hepatotoxicity, immune system suppression, developmental toxicity,  
53 and skin defects. TCDD mediates its toxicity by binding to the aryl hydrocarbon receptor  
54 (AhR) and subsequent alteration of the expression of target genes which exhibit dioxin  
55 response elements in their promoter moiety including the cytochrome CYP1A1 (Barouki *et al.*  
56 2007; Mimura and Fujii-Kuriyama 2003). Apart from phase I and phase II enzymes of the  
57 detoxification machinery, microarray studies allowed identifying chemokines as potential new  
58 target genes in liver (Boutros *et al.* 2009; Boutros *et al.* 2008) but also in testis (Rebourcet *et al.*  
59 2010), suggesting that TCDD exposure may induce inflammatory responses in addition to  
60 detoxifying processes. Evidences also accumulated showing that inflammatory cytokines  
61 including the tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) were downstream targets of AhR signalling  
62 pathways (Haarmann-Stemmann *et al.* 2009).

63 In the adult testis, Leydig cells, which are fully differentiated cells, produce the  
64 male primary steroid hormone, testosterone, in response to their trophic Luteinizing  
65 Hormone, LH. In addition to being under hormonal control, Leydig cells are under a local  
66 control exerted by autocrine and/or paracrine factors such as cytokines and chemokines  
67 (Benahmed 1997; Guazzone *et al.* 2009). Leydig cells are presumed to be somewhat resistant  
68 to various types of chemotoxicants due to the insufficiency of CYP1A1 expression and  
69 activity as demonstrated elsewhere (Chung *et al.* 2007). The xenobiotic metabolizing enzyme  
70 CYP1B1 can also be induced by treatment with AhR agonists (Shimada and Fujii-Kuriyama  
71 2004) and in contrast to CYP1A1, CYP1B1 is a predominantly extra-hepatic CYP enzyme.  
72 For example, it is highly expressed in adrenals and gonads (Leung *et al.* 2009). However, its  
73 regulation by PAHs in steroidogenic tissues is controversial (Deb *et al.* 2010; Mandal *et al.*  
74 2001).

75           In the present study, we were interested in delving further into the adverse effects  
76 caused by TCDD on testis physiology focusing on Leydig cells using a model of primary  
77 cultures. We demonstrated that Leydig cells expressed AhR, its repressor AhRR and Cyp1B1  
78 genes, and that they are under TCDD regulation. The chemokines Cxcl4 and Ccl5, previously  
79 identified *in vivo* as target genes for TCDD (Rebourcet *et al.* 2010) were directly (Cxcl4) or  
80 indirectly (Ccl5) regulated by TCDD. However, TCDD did not alter steroidogenic outcome  
81 of Leydig cells. Collectively, our data support the hypothesis that Leydig cells and thus the  
82 testis is not inert with respect to toxic insult. It also suggested that TCDD impacted the  
83 expression of target genes in Leydig cells under the complex network of hormones and  
84 cytokines.

85

## 86 **2. Materials and Methods**

### 87 *2.1. Leydig cell preparation*

88           Swiss male CD-1 mice aged of 8 weeks were purchased from Harlan Laboratories  
89 France (Gannat, France). Animals were killed by CO<sub>2</sub> asphyxia. Testes were removed and  
90 immediately used for cell preparations. All procedures were performed with the approval of  
91 the Regional Committee of Ethics for Animal Experiments.

92           Leydig cells were isolated and cultured in HAM's F12-DMEM (PAA Laboratories,  
93 Les Mureaux, France) at 32°C in an humidified atmosphere of 5% CO<sub>2</sub> as previously  
94 described (Carreau *et al.* 1988; Mazaud Guittot *et al.* 2008). Briefly, testes were decapsulated  
95 and digested with 0.25 mg/ml collagenase at 32°C for 10 min. The digestion procedure was  
96 stopped by dilution with fresh medium. Interstitial cells were collected in the supernatant and  
97 the pellet containing aggregates of Sertoli and germ cells was discarded. Interstitial cells were  
98 purified on a discontinuous Percoll density gradient (layers of 21, 26, 34, 40 and 60%  
99 Percoll). The gradient was centrifuged at 800 g for 30 min. The interface between 40 and 60%  
100 was collected and washed with medium to remove the Percoll. The presence of 3-β  
101 hydroxysteroid dehydrogenase (3beta-HSD) activity was revealed by a histochemical  
102 technique described in details elsewhere (Bilinska *et al.* 1997) using the anti-3beta-HSD  
103 antibody provided by Dr I. Mason (Reproductive and Developmental Sciences Division,  
104 Edinburgh, UK). It was used to determine the purity of Leydig cells (95%). Contamination by  
105 interstitial macrophages did not exceed 3%. Cells were resuspended in fresh culture medium  
106 supplemented with 2% Fetal Calf Serum (FCS). They were plated in 12-well plates (400,000  
107 cells/well). After 24 h, culture media were replaced with serum-free culture media.

108

## 109 *2.2 Leydig cell treatment*

110 Two days after plating, culture media were renewed and 0.5 ml of fresh serum-free culture  
111 media was added per well in the presence or not of different products listed below for the time  
112 periods indicated in the text. These products included human chorionic gonadotropin (hCG;  
113 100 ng/ml, Organon Schering-Plough, France) to mimic the effect of LH, dibutyryl cyclic  
114 AMP (dbcAMP; 0.2 mM, Sigma-Aldrich, France), recombinant Tumor Necrosis Factor alpha  
115 (TNF $\alpha$ ; 20 ng/ml; Peprotech France), Interleukin 1 alpha (IL1 $\alpha$ ; 20 ng/ml, Peprotech France),  
116 Lipopolysaccharides (LPS; 10 microg/ml; Sigma-Aldrich, France). Cytokines were used at a  
117 20 ng/ml concentration to maximally stimulate Leydig cells (Benahmed 1997; Hong *et al.*  
118 2004). For 2,3,7,8-tetrachlorodibenzo-p-dioxin (ref ED-901-C; LGC Promochem, Molsheim  
119 France), stock solutions were prepared in dimethylsulfoxide (DMSO) and appropriately  
120 diluted in culture medium. DMSO volume content per well was 0.04 microl in a final volume  
121 of 0.5ml per well. Cell viability was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-  
122 (3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] reduction method  
123 according to manufacturer's instructions (Promega, Charbonnières, France). Treatments did  
124 not alter cell viability which was over 95%. Doses of TCDD higher than 25 nM resulted in  
125 loss of cell viability and were not used in the present study.

126

## 127 *2.3 Testosterone Measurement by Enzyme Immunoassay*

128 Testosterone levels in the culture media were measured directly with a testosterone  
129 immunoassay kit purchased from Cayman Chemical Company (Interchim, Montlucon  
130 France). The sensitivity of the assay was 6 pg/ml.

131

## 132 *2.4 RNA extraction, Reverse Transcription and Quantitative PCR (RT-qPCR)*

133 Total RNA was extracted from cultured Leydig cells using TRI Reagent (Applied  
134 Bioystems, Courtaboeuf, France). RNA integrity was determined with the Agilent 2100  
135 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Massy, France). Briefly, First-  
136 strand cDNAs were synthesized from 1  $\mu$ g of total RNA in the presence of 100 U of  
137 Superscript II (Invitrogen, Eragny, France) and a mixture of random hexamers and oligo(dT)  
138 primers (Promega). Real-time PCR assays were performed in duplicates for each sample  
139 using a Rotor-Gene<sup>TM</sup> 6000 (Corbett Research, Mortlake, Australia), as described (Rebourcet  
140 *et al.* 2010). The list of the primers used (Invitrogen, Eragny, France) is available on Table 1.

141 Briefly, PCR was performed in the presence of 0.4  $\mu$ M of specific sense and antisense primers  
142 and 10  $\mu$ l Absolute QPCR SYBR Green ROX mix (Thermo Fisher Scientific, Courtaboeuf,  
143 France), in a total volume of 20  $\mu$ l. After the initial denaturation step of 15 min at 95°C, the  
144 reaction conditions were 40 cycles of 95°C for 15 sec, 55 to 62°C (depending on the primers,  
145 Table 1) for 10 sec, and 72°C for 20 sec. Melting curve analyses were performed immediately  
146 following the final PCR cycle to verify the specificity of the PCR product by checking its  $T_m$ .  
147 Rpl19 (ribosomal protein L19) gene was chosen for normalizing target genes in the testis. It  
148 was consistently and reproducibly expressed in all samples, and it did not vary following  
149 treatments including TCDD treatment. Relative quantification was made by the standard  
150 curve method for both target and housekeeping gene (endogenous control) in each sample. A  
151 series of dilutions of calibrator sample (external standard) was included in each experiment in  
152 order to generate an external standard curve. Then the concentration of the target in each  
153 sample was divided by the concentration of the housekeeping gene in each sample, to correct  
154 for sample heterogeneity and variability of detection.

155

## 156 *2.5 Statistical analysis*

157 All experiments have been performed at least three times, with independent preparations of  
158 cells. Data were expressed as means  $\pm$  SEM and the comparisons between treatments were  
159 made by one-way analysis of variance (ANOVA) followed by the post hoc Fisher PLSD test  
160 for multiple comparisons. A  $p$  value of less than 0.05 was considered significant. All  
161 statistical analyses were done with the aid of Statview 5.0 software package (SAS Institute  
162 Inc. Cary, NC 27513).

163

## 164 **3. Results**

### 165 *3.1 Steroidogenic outcome of Leydig cells treated with TCDD*

166 Addition of TCDD (25 nM) to Leydig cells did not alter basal and dbcAMP-induced  
167 testosterone production after 6 h of treatment (Fig. 1a). RT-qPCR experiments were also  
168 developed to survey expression levels of genes that mediate the first steps in steroidogenesis  
169 including the Steroidogenic acute regulatory protein (StAR) and Cyp11a1 in Leydig cells  
170 treated with TCDD, and stimulated or not with hCG or dbcAMP for 6 h. Within these  
171 conditions, TCDD had no impact on StAR (Fig. 1b) or Cyp11a1 (data not shown) gene  
172 expression levels.

173

174 *3.2 Gene expression of AhR, the repressor AhRR and Cyp1b1, and their regulation by TCDD*  
175 *and hCG or dbcAMP*

176 TCDD mainly exerts its action through binding to AhR. This step is followed by a  
177 rapid activation of target genes including the repressor AhRR, Cyp1A1 and Cyp1B1 (Abel  
178 and Haarmann-Stemmann 2010; Barouki *et al.* 2007). Cyp1A1 is not expressed at all in the  
179 testis (Chung *et al.* 2007; Rebourcet *et al.* 2010) nor was it induced following TCDD  
180 challenging in Leydig cells (data not shown). We found that Leydig cells expressed AhR, the  
181 repressor AhRR and Cyp1b1. TCDD added for 6 h regulated significantly ( $p < 0.05$ ) the level  
182 of mRNAs encoding AhR (a 40% decrease), AhRR (a 7 fold-increase) and Cyp1b1 (a 2.5  
183 fold-increase) (Fig. 2). Addition of hCG 100 ng/ml or dbcAMP 0.2 mM had no effect on AhR  
184 and Cyp1b1 gene expression levels (Fig. 2). However, hCG significantly halved the TCDD-  
185 induced increase in Ahrr gene expression levels after 6 h of treatment (Fig. 2b). This effect  
186 was also observed after 24 h of treatment with a 4-fold decrease in hCG and TCDD co-treated  
187 cells versus TCDD-treated cells (data not shown). Addition of dbcAMP also opposed TCDD  
188 enhancement of AhRR gene expression levels after 6 h of treatment, indicating the  
189 involvement of Protein Kinase A (Fig. 2b).

190

191 *3.3 Expression of the chemokines Ccl5 and Cxcl4 in primary culture of Leydig cells and their*  
192 *regulation by TCDD*

193 We previously demonstrated that Ccl5 and Cxcl4 were 2 chemokines whose  
194 expression levels were altered in rat testis exposed to TCDD at gestational age 15. In addition,  
195 CCL5 immuno-reactivity was mostly confined to Leydig cells (Rebourcet *et al.*, 2010). We  
196 therefore determined by RT-qPCR that the chemokines Ccl5 and Cxcl4 were expressed at the  
197 mRNA level in the mouse cultured Leydig cells. In addition, we found that Cxcl4 (Fig. 3a)  
198 but not Ccl5 (Fig. 3b) gene expression levels were significantly up-regulated by TCDD and  
199 dbcAMP. Combined treatment resulted in additive effects (Fig. 3a).

200

201 *3.4 Local regulation of the TCDD targeted genes by TNF $\alpha$*

202 We investigated potential interactions between TNF $\alpha$  and TCDD in the model of  
203 primary culture of Leydig cells. Cells were treated for 24 h. We found that TNF $\alpha$  stimulated  
204 significantly ( $p < 0.05$ ) AhR but not AhRR gene expression levels (Fig. 4). The negative  
205 effects of TCDD on AhR gene expression levels observed at 6 h of treatment (Fig. 2a) were

206 not observed at 24 h of treatment. However, TCDD-treated cells did no longer respond to  
207 TNF $\alpha$  by enhanced AhR mRNA levels (Fig. 4a). Gene expression levels of Cxcl4 and Ccl5  
208 were also investigated. The addition of TNF $\alpha$ , IL1 $\alpha$  and LPS, described as stimulators of  
209 chemokines (Graves and Jiang 1995; Nomiya *et al.* 2010) up-regulated Cxcl4 (Fig. 5a) and  
210 Ccl5 (Fig. 5b) gene expression levels in primary culture of Leydig cells. TNF $\alpha$  was a potent  
211 stimulator for the two chemokines (4 and 4.4-fold increase for Cxcl4 and Ccl5 respectively,  
212  $p < 0.05$ ) (Fig. 5). By contrast, IL1 $\alpha$  stimulated more Cxcl4 (7-fold increase) than Ccl5 (1.8-  
213 fold-increase) gene expression. LPS stimulated more Ccl5 (8-fold increase) than Cxcl4 (2.8-  
214 fold increase) gene expression (Fig. 5). Extending data presented in Fig. 3, we observed that  
215 TCDD could significantly ( $p < 0.05$ ) oppose the TNF $\alpha$ -induced increase in Ccl5 mRNA levels  
216 (Fig. 5b); with no effect on TNF $\alpha$ -induced increase in Cxcl4 mRNA levels (Fig. 5a). A  
217 summary of all the observed effects is provided on Table 2.

218

#### 219 4. Discussion

220 Understanding the detrimental effects caused by TCDD is limited using animal  
221 models, and primary cell culture models represent useful tools to decipher mechanistic  
222 toxicities. In the present study, we were interested in delving further into the adverse effects  
223 caused by TCDD on Leydig cell physiology focusing on AhR dependent genes and  
224 chemokines, recently identified as TCDD target genes.

225 We first demonstrated that TCDD did not impact the steroidogenic outcome of Leydig  
226 cells assessed through measurement of StAR and Cyp11a1 expression levels, and testosterone  
227 production in Leydig cells cultured in basal conditions and under stimulation by the trophic  
228 hormone or dbcAMP. These data are consistent with previous *in vitro* studies showing no  
229 direct effect of TCDD on steroidogenesis in MA-10 mouse Leydig tumor cells and adult rat  
230 Leydig cells (Mandal *et al.*, 2001). These data are also consistent with our previous study  
231 indicating no change in testosterone levels in the testes of rats exposed *in utero* to low doses  
232 of TCDD (Rebourcet *et al.*, 2010), whereas reduced testosterone levels were observed in adult  
233 rats treated with very high doses of TCDD (Johnson *et al.* 1994; Kleeman *et al.* 1990; Moore  
234 *et al.* 1985).

235 Leydig cells recovered from fully mature animals are known to express AhR  
236 (Schultz *et al.* 2003), but no reports have yet identified the TCDD regulation of AhR in  
237 Leydig cells nor determined if the repressor AhRR was present in Leydig cells and regulated  
238 by TCDD. We found that TCDD down-regulated transiently the expression of AhR gene, and

239 enhanced the expression of AhRR gene, suggesting negative feedback to limit AhR signalling  
240 effect. Indeed, it has been previously shown that AhRR functions as a naturally occurring  
241 dominant-negative factor limiting AhR signalling pathway (Abel and Haarmann-Stemmann  
242 2010; Mimura *et al.* 1999). Hence, AHRR is an important determinant of tissue specific  
243 responsiveness to TCDD, and strong evidences have been reported on an inverse relationship  
244 between AHRR expression and sensitivity to induction of xenobiotic-metabolizing enzymes  
245 caused by TCDD (Korkalainen *et al.* 2004). The data presented herein i.e., high expression of  
246 AhRR and no detectable Cyp1a1 induction are in keeping with these observations.  
247 Interestingly, in conditions of increased gene expression levels of StAR following hCG or  
248 dbcAMP stimulation, genes encoding AhR and AhRR had unchanged expression levels. We  
249 next investigated the combined effects of TCDD with hCG/dbcAMP or TNF $\alpha$ . Indeed, TNF $\alpha$   
250 may originate from the systemic circulation or from the neighbouring interstitial macrophages  
251 (Guazzone *et al.*, 2009). We found that addition of hCG which protects testis function against  
252 TCDD in adult rats (Lai *et al.* 2005), while not acting directly on AhR or AhRR gene  
253 expression levels, attenuated the TCDD-induced stimulation of AhRR. Unlike hCG, TNF $\alpha$   
254 did not reduce TCDD action on the AhRR gene expression levels, and enhanced AhR  
255 expression levels. Altogether, these data are in keeping with previous studies showing that  
256 gonadotrophins and TNF $\alpha$  exert inverse effects on Leydig cells (Benahmed 1997; Guazzone  
257 *et al.* 2009).

258 Testicular Cyp1B1 is localized predominantly in Leydig cells (Ge *et al.* 2005) and  
259 subject to developmental regulation in rat testis (Leung *et al.* 2009). Herein, we found that  
260 TCDD enhanced Cyp1b1 expression levels in Leydig cells but hCG and dbcAMP had no  
261 effect. These data do not support previous studies (Deb and Bandiera 2011; Deb *et al.* 2010).  
262 Such a discrepancy may result from the use of primary cultures of adult Leydig cells in our  
263 system versus Leydig cell lines in Deb and collaborators. In addition, adult Leydig cells are  
264 less responsive to gonadotropins with aging (Midzak *et al.* 2009) and, Deb and collaborators  
265 (Deb *et al.* 2010) also observed that *in vivo* treatment with TCDD increased testicular  
266 CYP1B1 protein levels in adult rats. The meaning of the enhancement of Cyp1b1 by TCDD  
267 is unclear. TCDD is not metabolized by CYP1B1. Besides, CYP1B1 is not required for  
268 mammalian development, reproductive vitality and physiological homeostasis as  
269 demonstrated by the absence of major phenotype in Cyp1b1-null mice (Buters *et al.* 1999).  
270 More studies are required to elucidate the role CYP1B1 may play in Leydig cell physiology.

271 It has been recently demonstrated that TCDD apart from the classical battery of  
272 genes of the detoxification machinery, may provoke inflammatory adverse effects  
273 (Haarmann-Stemmann *et al.* 2009). For example, it was demonstrated that TCDD could  
274 enhance TNF $\alpha$  secretion in a model of cultured adipocytes (Nishiumi *et al.* 2010) as well as  
275 in the serum of rats orally treated with TCDD (Ciftci *et al.* 2010). It was also shown that  
276 dioxin through binding to AhR could stimulate Ccl5 gene expression in an *in vitro* model of  
277 endometriosis (Yu *et al.* 2008; Zhao *et al.* 2002), Ccl1, a chemokine involved in  
278 cardiovascular diseases and inflammatory or allergic processes (N'Diaye *et al.* 2006) and Ccl2  
279 in multiple organs of mice (Vogel *et al.* 2007). TCDD also inhibited Cxcl12 gene expression  
280 and its receptor Cxcr4 in MCF-7 breast cancer cells (Hsu *et al.* 2007). Chemokines are a  
281 subset of small chemotactic cytokines produced by both immuno-competent cells and non-  
282 immune cells. They are secondary pro-inflammatory mediators that are induced by cytokines  
283 such as interleukin-1 or TNF $\alpha$  (Graves and Jiang 1995; Nomiya *et al.* 2010). We here  
284 found that Leydig cells could express Ccl5 and Cxcl4, and that these chemokines responded  
285 to stimulation by LPS, IL1 $\alpha$  and TNF $\alpha$ . In addition, depending on the conditions (presence or  
286 not of TNF $\alpha$ ), these two chemokines were targeted by TCDD in primary cultures of adult  
287 mouse Leydig cells, consistently with our previous *in vivo* findings (Rebourcet *et al.*, 2010).

288 Collectively, we demonstrated that TCDD can directly and indirectly impact in  
289 *in vitro* adult Leydig cell function through alteration in the gene expression levels of chemokines  
290 as well as AhR signalling pathway, supporting the hypothesis that Leydig cells and thus the  
291 testis are not inert with respect to toxic insult. If considering that TCDD may stimulate TNF $\alpha$   
292 secretion, our data raise the hypothesis that the resulting AhR activation in testis following an  
293 *in vivo* TCDD insult might be the sum of a direct action by TCDD itself on Leydig cells and  
294 an indirect inflammatory response with elevated systemic TNF $\alpha$  subsequently activating AhR  
295 signalling pathway in Leydig cells. More insight into the molecular mechanisms behind the  
296 influence of TCDD on Leydig cells will require determining the involvement of the TNF $\alpha$   
297 signalling transduction pathways, specifically the involvement of NF- $\kappa$ B and mitogen-  
298 activated protein kinases (MAPKs). Indeed, and depending on cell types and culture  
299 conditions, it was shown that hCG could attenuate NF- $\kappa$ B activation and cytokine expression  
300 (Huber *et al.* 2007), and trigger transient MAPK kinase activation (Brion *et al.* 2011).  
301 Therefore, integrated responses to TCDD would be highly dependent on the cell type, its local  
302 and hormonal environment.

303

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312 BLM and DN designed the study. DN, MAC and BLM performed the cultures and treatment.  
313 NV, MV, EL performed the QPCR dosages. DR performed the immunostaining and cell  
314 viability tests. AJ and MV performed the western blotting experiments. BLM, DN and MB  
315 analyzed the data and wrote the paper. All authors read and approved the final manuscript.

316

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**Table 1: List and sequence of primers used for PCR analysis**

Gene symbol	Accession no.	forward 5'-3'	Reverse 5'-3'	Size (bp)	Hybrid. T(°C)
<b>RPL19</b>	NM_009078.2	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC	195	58
<b>CCL5</b>	NM_031116.3	CTTGCAGTCGTCTTTGTAC	GACTAGAGCAAGCAATGACAG	158	58
<b>CXCL4 (PF4)</b>	NM_019932.4	AGCGATGGAGATCTTAGCTGTGTG	GGTCCAGGCAAATTTCTCCCATTC	160	58
<b>AHR</b>	NM_013464.4	TCATCTGGTTTCCTGGCAATGAAT	ATAAGCTGCCCTTGGCATC	245	62
<b>AHRR</b>	NM_009644.2	TCAGGGGACAAACAGATAGG	CTCAAGTGTACTGGTGTCTC	220	55
<b>CYP1B1</b>	NM_012940.1	GCAGCCGCCTTCTGGTAGC	CCACGCGCCCTGTCCCTACT	116	60
<b>STAR</b>	NM_000349	ATGCAGAAGGCCTTGGGCAT	AACACCTTGCCACATCTGG	113	60
<b>CYP11A1</b>	NM_019779	ACAAGTGCCCTTCAAGAAC	TCCTTGATGCTGGCTTTGAG	223	58

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The size of the expected PCR fragment in base pairs (bp) and the hybridization temperature (Topt) for annealing are reported.

**Table 2: Summary of the effects described**

Genes surveyed	Stimuli added in vitro		
	TCDD	hCG/dbcAMP	TNF $\alpha$
<b>Star and Cyp11a1</b>	no effect	strong up-regulation	not determined
<b>AhR</b>	down-regulation at 6 h, not at 24 h; TCDD opposed TNF $\alpha$ -effect on AhR at 24 h	no effect	up-regulation at 24 h
<b>AhRR</b>	up-regulation at 6 & 24 h	no effect per se; can oppose TCDD-effect on AhRR at 6 & 24 h	no effect
<b>Cyp1b1</b>	up-regulation at 6 h	no effect	not determined
<b>Cxcl4</b>	up-regulation at 24 h	up-regulation at 24 h	up-regulation at 24 h
<b>Ccl5</b>	no effect per se; can oppose TNF $\alpha$ -effect at 24 h	no effect	up-regulation at 24 h

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476 **FIGURE LEGENDS:**

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478 **Figure 1:** Testosterone production (a) and relative gene expression of StAR (b) in primary  
479 cultures of Leydig cells recovered from 8-week old mouse testes treated for 6 h with various  
480 factors and assessed by EIA and RT-qPCR analysis, respectively. Factors added included  
481 TCDD 25 nM, dbcAMP (0.2 mM) and hCG 100 ng/ml. Testosterone production is expressed  
482 in ng/ml. StAR levels were normalized using Rpl19 and are expressed relatively to controls  
483 arbitrarily fixed to 1. Values are the mean  $\pm$  SEM of n= 4 separate experiments. a,  $p<0.05$   
484 versus control. Boxes in black correspond to controls and boxes in grey indicate treatment  
485 with TCDD. Small window highlights testosterone production in basal conditions.

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487 **Figure 2:** Relative expression of Ahr (a), Ahrr (b) and Cyp1b1 (c) in primary cultures of  
488 Leydig cells recovered from 8-week old mouse testes treated for 6 h with various factors and  
489 assessed by RT-qPCR analysis. Factors added included TCDD (25 nM), hCG (100 ng/ml), or  
490 dbcAMP (0.2 mM). Ahr (a), Ahrr (b) and Cyp1b1 (c) levels were normalized using Rpl19 and  
491 are expressed relatively to controls arbitrarily fixed to 1. Values are the mean  $\pm$  SEM of n= 4  
492 separate experiments. a,  $p<0.05$  versus its respective control; b,  $p<0.05$  versus TCDD-treated  
493 samples in (b). Boxes in black correspond to controls and boxes in grey indicate treatment  
494 with TCDD.

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496 **Figure 3:** Relative expression of Cxcl4 (a) and Ccl5 (b) in primary cultures of Leydig cells  
497 recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by  
498 RT-qPCR analysis. Factors added included TCDD (25 nM), dbcAMP (0.2 mM) or the two.  
499 Cxcl4 (a) and Ccl5 (b) levels were normalized using Rpl19 and are expressed relatively to  
500 controls arbitrarily fixed to 1. Values are the mean  $\pm$  SEM of n= 6 separate experiments. a,  
501  $p<0.05$  versus its respective control; b,  $p<0.05$  versus dbcAMP and TCDD-treated cells.  
502 Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD.

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504 **Figure 4:** Relative expression of Ahr (a) and Ahrr (b) in primary cultures of Leydig cells  
505 recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by  
506 RT-qPCR analysis. Factors added included TCDD (25 nM), TNF  $\alpha$  (20 ng/ml) or the two.  
507 Ahr and Ahrr levels were normalized using Rpl19 and are expressed relatively to controls  
508 arbitrarily fixed to 1. Values are the mean  $\pm$  SEM of n= 8 separate experiments. a,  $p<0.05$

509 versus its respective control; b,  $p < 0.05$  versus TCDD or TCDD and TNF  $\alpha$ -treated cells in  
510 (b). Boxes in black correspond to controls and boxes in grey indicate treatment with TCDD.

511

512 **Figure 5:** Relative expression of Cxcl4 (a) and Ccl5 (b) in primary cultures of Leydig cells  
513 recovered from 8-week old mouse testes treated for 24 h with various factors and assessed by  
514 RT-qPCR analysis. Factors added included LPS (10  $\mu\text{g/ml}$ ),  $\text{IL1}\alpha$  or TNF  $\alpha$  (20  $\text{ng/ml}$ ), or  
515 TCDD (25 nM) or TNF $\alpha$  and TCDD in co-treatment. Cxcl4 (a) and Ccl5 (b) levels were  
516 normalized using Rpl19 and are expressed relatively to controls arbitrarily fixed to 1. Values  
517 are the mean  $\pm$  SEM of  $n = 9$  separate experiments. a,  $p < 0.05$  versus its respective control. B,  
518  $p < 0.05$  versus TNF $\alpha$  –treated cells in (b). Boxes in black correspond to controls and boxes in  
519 grey indicate treatment with TCDD.

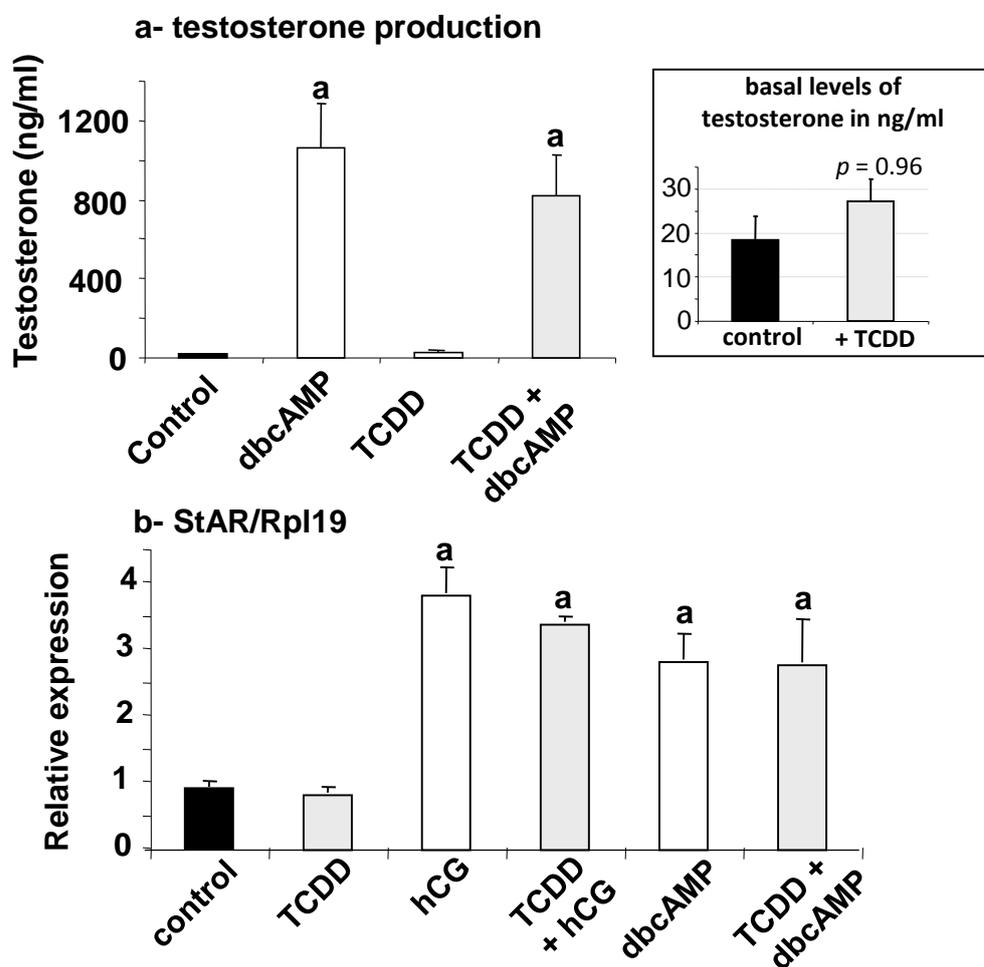
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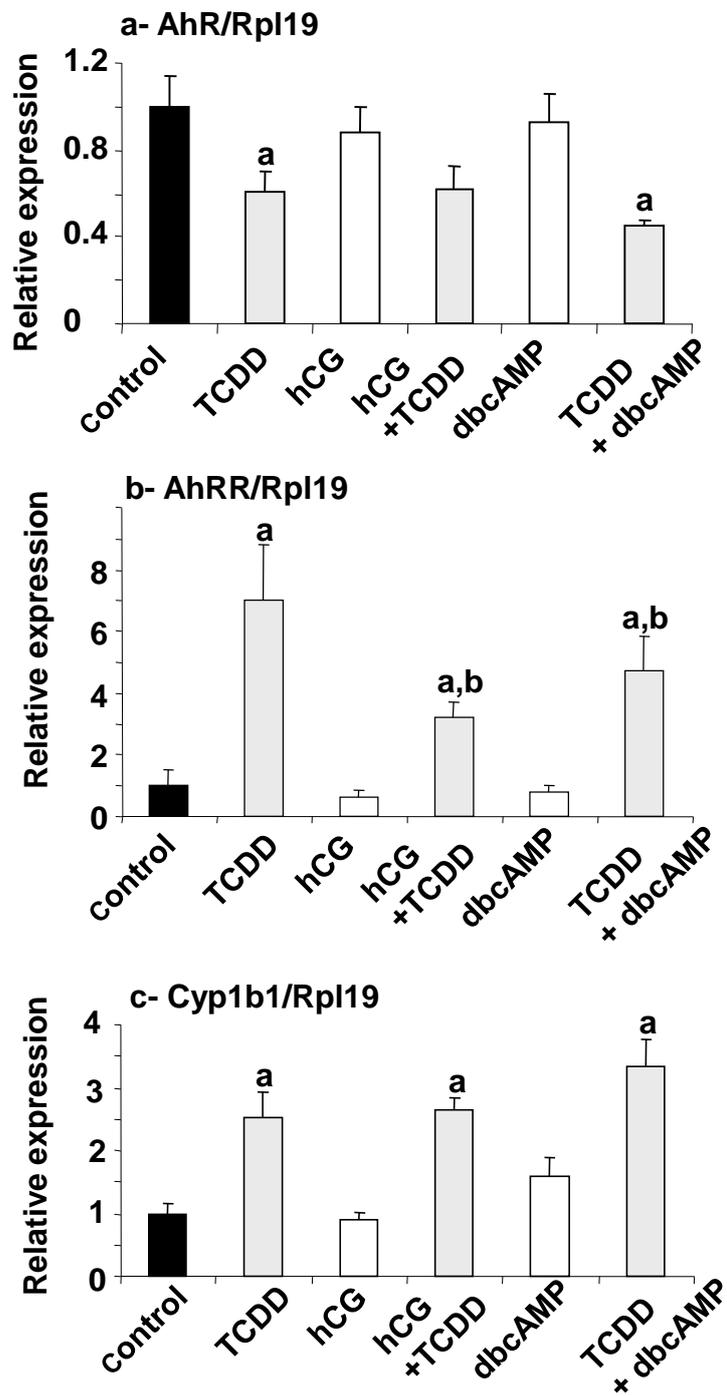
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Figure 1



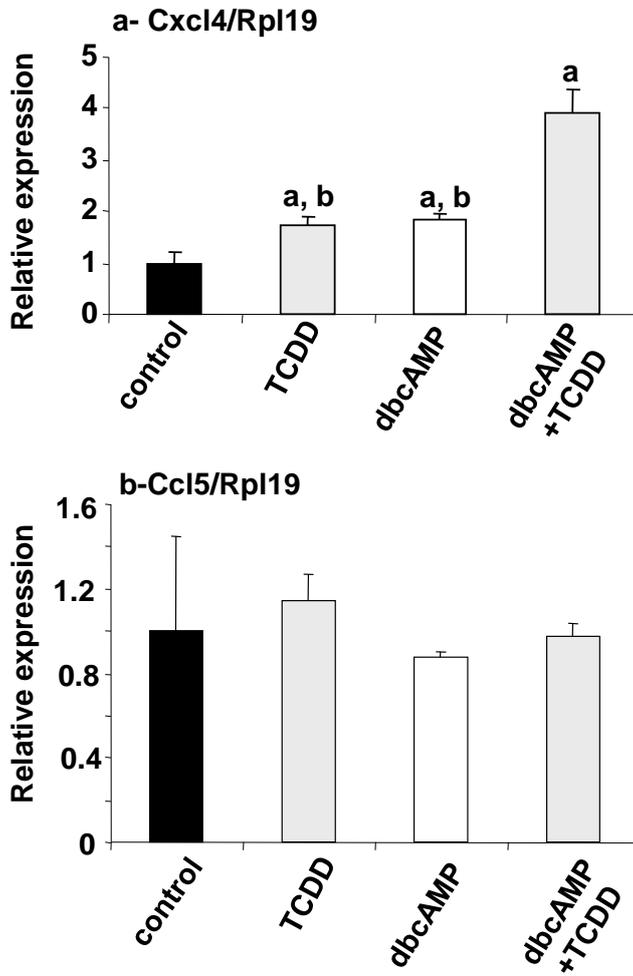
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Figure 2



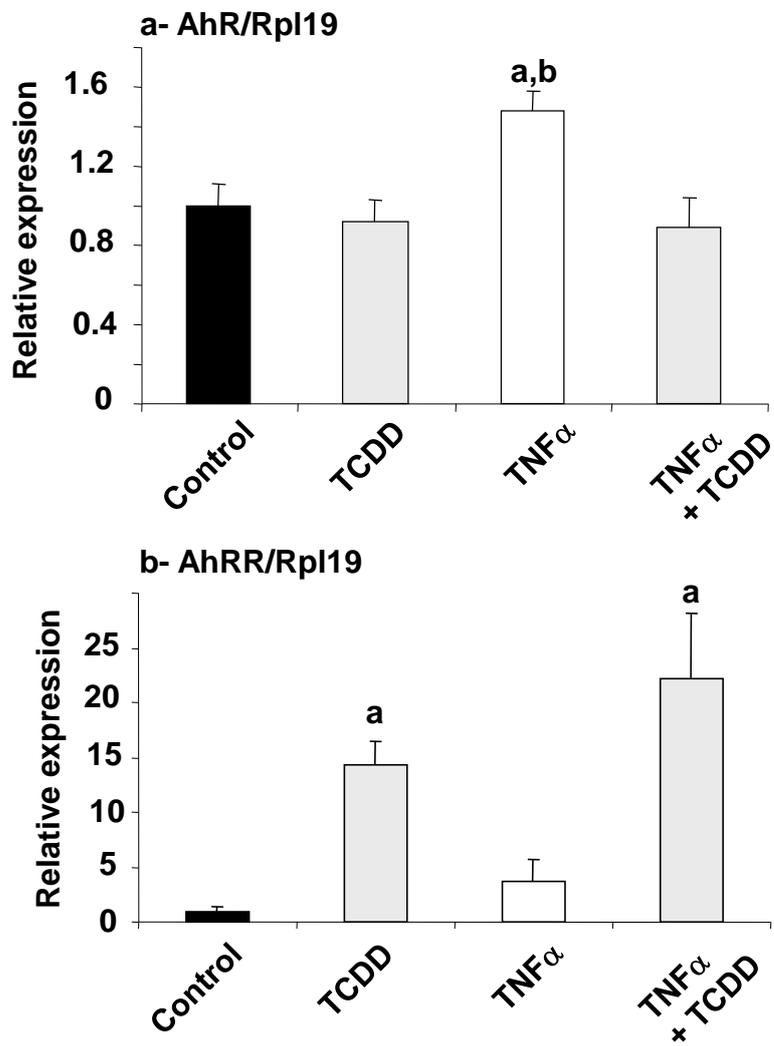
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Figure 3



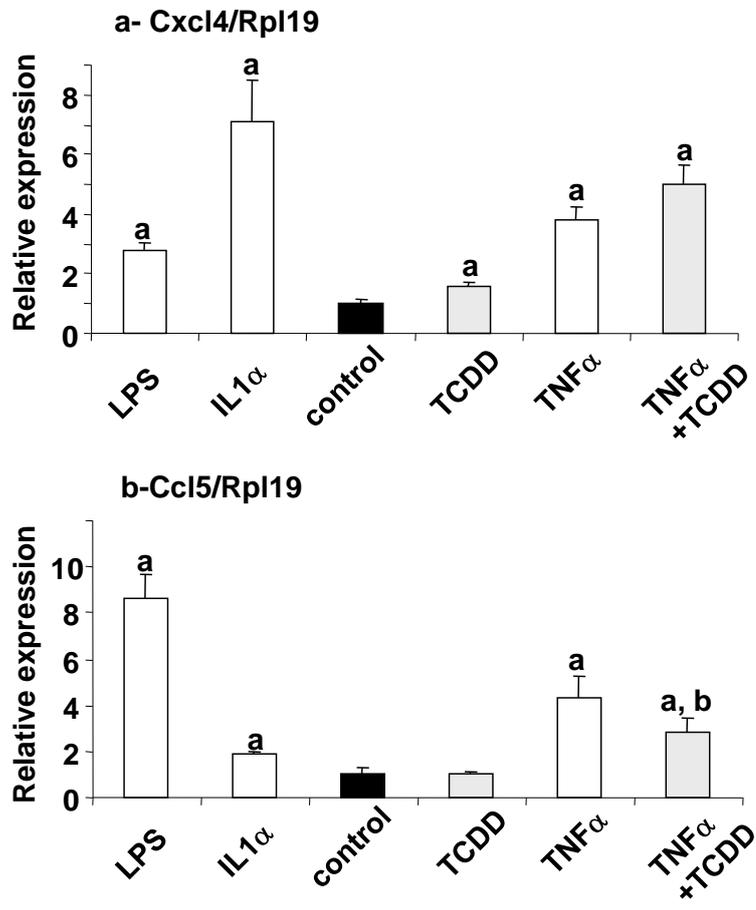
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Figure 4



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Figure 5



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