

SHORT REPORT: 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) REDUCES *LEISHMANIA MAJOR* BURDENS IN C57BL/6 MICE

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Abstract. Acute exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can suppress adaptive immunity. In this study, pre-exposure of *Leishmania major*-infected mice to TCDD caused a dose-dependent and unexpected decrease in parasite burdens on day 20 after infection. In contrast, TCDD-mediated lymphoid atrophy, suppressed antibody levels, and enhanced interleukin-2 production were observed as expected. These results suggest that TCDD may enhance resistance to *L. major* in the face of immune suppression.

Leishmania major is an obligate intracellular parasite of mammals that resides within parasitophorous vacuoles of phagocytic cells, primarily macrophages. Most strains of domestic mice successfully resist primary subcutaneous infection with *L. major* by the production of parasite-killing NO within infected macrophages. This resistance is dependent on the development of *L. major*-specific CD4⁺ Th1 cells^{1–3} but does not require B cells or CD8⁺ T cells.^{4,5} In contrast, BALB/c mice that are infected in the same way will fail to control *L. major* growth because of Th2 responses and insufficient interferon (IFN)- γ -induced macrophage activation.^{1–3}

The aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a long-lived, non-metabolized environmental toxicant that is lipophilic and resistant to metabolic degradation, properties that facilitate bioaccumulation in animals.^{6,7} TCDD exposure can cause multiple toxic responses in humans and animals to varying degrees. These toxic responses include chloracne, teratogenesis, altered metabolism, carcinogenicity, and immunotoxicity. TCDD immunotoxic effects include lymphoid atrophy, especially of the thymus, and suppression of adaptive immunity (cellular and humoral), a response that has been observed in every experimental animal tested.^{8,9} TCDD toxicity is generally dependent on ligation of TCDD to the aryl hydrocarbon (Ah) receptor.^{9,10} Immunotoxic mechanisms downstream of the Ah receptor are still unclear but may include altered cytokine or hormone production, altered costimulation, altered phosphorylation activity, altered intracellular calcium homeostasis, and induction of apoptosis.^{8,9,11–14}

The initial goal of this study was to use *L. major*-infected mice to explore the impact of TCDD on the development of a primary CD4⁺ T-cell response. To our knowledge, this is the first report of the effects of TCDD in *L. major*-infected mice. We hypothesized that TCDD exposure would suppress the development of resistant Th1 responses in *L. major*-infected C57Bl/6 mice, resulting in greater parasite burdens.

C57Bl/6 mice were originally obtained from Jackson Laboratories (Bar Harbor, ME) and used to establish a breeding

colony to provide animals for these studies. The maintenance and care of all experimental animals complied with National Institutes of Health guidelines for the humane use of laboratory animals. TCDD was prepared in peanut oil for oral administration to mice as previously described.¹⁴ Female mice (6–8 weeks of age) were given peanut oil or TCDD 1 day before infection with 10⁶ *L. major* promastigotes (LV39, RHO/SU/59/P, Neal, or P strain) by subcutaneous injection into one rear foot pad.¹⁵

After 20 days of infection, typical evidence of TCDD-mediated immune suppression was observed. As shown in Table 1 (line 1), TCDD exposure at 40 μ g/kg caused a significant decrease in thymus weights (normalized to body weights) by 22% relative to controls ($P = 0.022$). TCDD treatment (40 μ g/kg) also significantly reduced the numbers of viable cells (measured by trypan blue exclusion) per lesion-draining popliteal lymph node by 47% relative to controls (Table 1, line 6; $P = 0.046$). The levels of serum antibodies specific to soluble *L. major* antigens were measured using an ELISA¹⁶ with a phosphatase-conjugated secondary goat anti-mouse Ig antibody (KPL, Gaithersburg, MD). At a serum dilution of 100:1, *L. major*-specific antibody levels from TCDD-treated mice (40 μ g/kg) were ~37% of control levels (Table 1, line 13). These results confirmed previously reported effects of TCDD.^{8–14}

In contrast to our prediction, TCDD exposure did not cause an increase in parasite burdens. Foot parasite burdens were determined by limiting dilution analysis as previously described.¹⁷ As shown in Figure 1A, on day 20 after infection, a dose-dependent decrease in the number of viable *L. major* was observed per foot in TCDD-treated mice. Exposure to TCDD at the highest dose (40 μ g/kg) resulted in a significant decrease in viable *L. major* numbers by ~10-fold when compared with control mice (Figure 1B). In addition, TCDD exposure at 40 μ g/kg significantly delayed the resolution of foot lesions (Figure 1C). Lesion size was monitored over time with vernier calipers (lesion size = infected foot – contralateral uninfected foot). The lesions of all infected animals eventually resolved, regardless of treatment, and the numbers of viable *L. major* in infected feet or lesion-draining lymph nodes on day 139 after infection were < 130 (data not shown). These results indicate that TCDD did not prevent a resistant outcome of *L. major* infection in C57Bl/6 mice.

Coordinated cytokine production is essential for control of

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TABLE 1
TCDD treatment decreased lymphoid cell numbers and increased IL-2 production at 20 days after *L. major* infection

Line		TCDD treatment ($\mu\text{g/kg}$)		<i>P</i> *
		0	40	
1	Thymus/body weight (mg/g)†	4.02 \pm 0.19	3.13 \pm 0.19	0.022
2	Popliteal lymph node cells			
3	CD4 ⁺ (%)†	18 \pm 0	20 \pm 2	
4	CE8 ⁺ (%)†	13 \pm 0	13 \pm 1	
5	B220 ⁺ (%)†	67 \pm 1	65 \pm 3	
6	Total cells ($\times 10^{-6}$)‡	112.8 \pm 13.1	59.4 \pm 15.1	0.046
7	<i>Ex vivo</i> cytokine production§			
8	IL-2 (pg/mL at 24 hours)‡	34.8 \pm 1.8	66.6 \pm 2.1	< 0.001
9	IL-4 (pg/mL at 24 hours)‡	6.2 \pm 1.7	< 1.5**	
10	IL-5 (pg/mL at 48 hours)‡	49.4 \pm 17.5	37.0 \pm 21.4	
11	IL-10 (pg/mL at 48 hours)‡	844 \pm 146	582 \pm 179	
12	IFN- γ (ng/mL at 48 hours)‡	79.1 \pm 6.8	82.0 \pm 8.4	
13	Serum anti- <i>L. major</i> antibody levels¶	100%	36.7%	ND††

* Values of *P* if < 0.05.

† Data are representative of two to three experiments and are presented as means \pm SEM.

‡ Data are presented as least square means \pm SEM for two to three experiments analyzed together by two way ANOVA.

§ *Ex vivo* cultures were established as previously described.²¹ Cytokine levels represent *L. major*-stimulated production minus spontaneous production for each experimental sample.

¶ Data are representative of two experiments and are presented as percent of control for pools of three to five animals per group.

** Indicates the mean sample concentration was below the limit of detection for the assay.

†† ND, not determined.

L. major, and TCDD has been previously shown to alter antigen-driven cytokine production in mice.^{14,18–20} In particular, interleukin (IL)-2 expression can be directly upregulated by TCDD through activation of the Ah receptor.¹¹ *Ex vivo* cytokine production was examined in this study on day 20 after *L. major* infection. Cytokines in supernatants from cultures of *L. major*-restimulated popliteal lymph node cells were measured by ELISA.²¹ As shown in Table 1 (line 8), for cells taken from TCDD-treated mice, the levels of IL-2 in culture were significantly higher (by 91%) than those of controls (*P* < 0.001). In contrast, no significant TCDD-mediated change in IL-4, IL-5, IL-10, or IFN- γ levels were found (Table 1, lines 9–12). In addition, no significant changes in lymphocyte differentials were observed in the popliteal lymph nodes of TCDD-treated mice (CD4⁺, CD8⁺, or B220⁺ cells, measured by flow cytometry²²; Table 1, lines 3–5).

TCDD is not known to cause generalized activation of macrophages, but some changes in macrophage function have been reported. These include increased superoxide production,^{23,24} increased tumor necrosis factor (TNF)- α production,^{25,26} reduced endocytosis and adherence,²⁷ and reduced B7 expression.¹³ In contrast, measurable changes in apoptosis,²⁷ phagocytosis, or tumor cell killing^{28,29} have not been observed. One explanation for the reduced parasite numbers observed in this study (Figure 1) may be the altered cytokine environment. Nacy and others³⁰ showed that IL-2 can act as a co-factor, along with IFN- γ , to enhance the NO-mediated killing of *L. major* by macrophages. Importantly, Nacy and others³⁰ also showed that enhanced TNF production by IL-2 + IFN- γ -treated macrophages was an essential intermediate step leading to enhanced parasite killing. Thus, TCDD-enhanced IL-2 production and normal IFN- γ production (Table 1), coupled with enhanced TNF production,^{31,32} may enhance NO production, leading to reduced parasite numbers. The role of TNF in reducing parasite burdens in this experimental model will be explored in future studies.

TCDD has been shown to suppress the immune responses of mice to a variety of infectious agents including *Listeria monocytogenes*, *Plasmodium yoelii*, murine influenza virus, and others.^{18,33,34} TCDD-suppressed resistance to experi-

mental infection can result in increased disease severity and mortality.^{9,18,32} We believe that the data presented here are unique in suggesting enhanced resistance to *L. major* in TCDD-treated mice, which simultaneously displayed some of the classic signs of TCDD-induced adaptive immune suppression. TCDD is unlikely to cause reduced *L. major* burdens through direct toxicity to the parasite because, although ligand-activated Ah receptors are known in metazoans, they are not known in protists.³⁵ Thus, any direct toxicity of TCDD in *Leishmania* would likely be caused through a different mechanism. The authors are unaware of any published studies of the effects of TCDD in protozoans. Preliminary studies in this laboratory have found that *L. major* proliferation and infectivity were unchanged when the parasites were cultured in the presence of TCDD at concentrations up to 5×10^{-7} mol/L (data not shown), a concentration that is higher than would be expected in the skin of a mouse given TCDD at 40 $\mu\text{g/kg}$.³⁶ Therefore, the impact of TCDD on *L. major* burdens in mice is probably caused by the effects of TCDD on mice alone.

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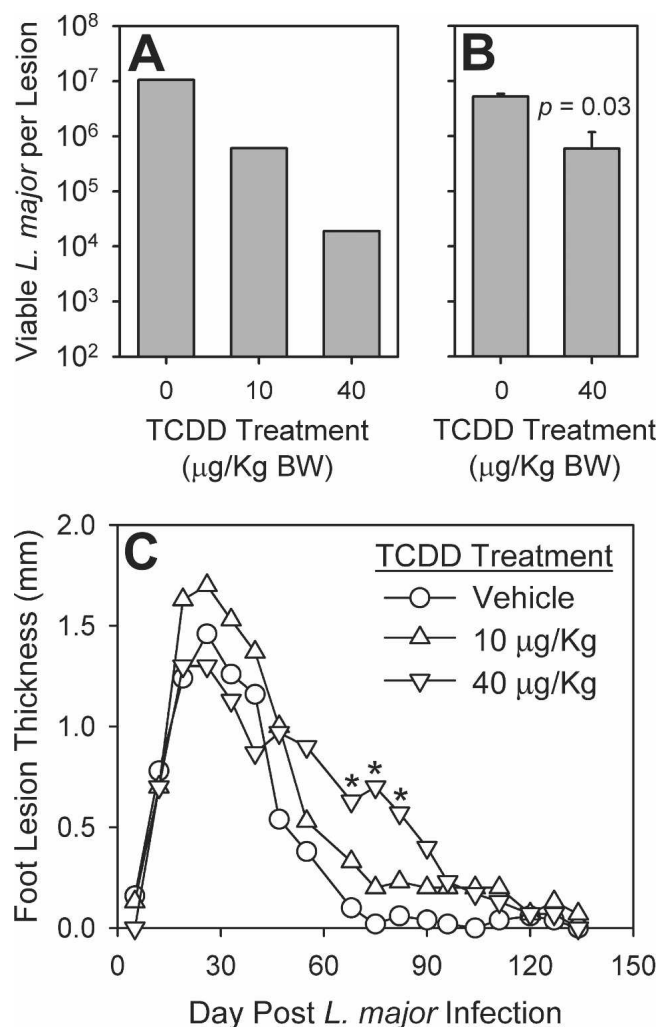


FIGURE 1. TCDD treatment decreased *L. major* burdens in foot lesions while delaying the resolution of those lesions. Peanut oil or TCDD were given orally 1 day before infection with 10^6 *L. major* promastigotes in the left rear foot pad.^{10,11} Parasite burdens¹² were determined on day 20 after infection for pools (two to three mice per pool) of homogenized feet. **A**, Results from one dose-response experiment with one to two pools of feet per treatment group are shown. **B**, Results from three independent experiments are shown combined. Data were analyzed by two-way ANOVA and are presented as least square means \pm SEM for the effect of TCDD. **C**, Lesion thickness over time is shown as mean \pm SEM for three to five mice per treatment group. Data were analyzed by repeated-measure two-way ANOVA followed by Tukey all-pairwise comparison. *Statistically significant difference from control ($P < 0.05$).

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