

**ASPECTS OF DIOXIN TOXICITY ARE MEDIATED BY
INTERLEUKIN 1-LIKE CYTOKINES.**

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Abbreviations: IL1, interleukin 1; dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AHR, aryl hydrocarbon receptor; PAS, Period-ARNT-Single-minded; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; ARNT, AHR nuclear translocator; ALT, alanine aminotransferase; TUNEL, TdT-mediated dUTP nick-end labeling; DRE, dioxin response elements; CYP, cytochrome P450-dependent monooxygenases; IL6, interleukin 6.

ABSTRACT:

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) results in a broad spectrum of toxic effects. Most, if not all, of these responses are dependent upon the binding of dioxin to the aryl hydrocarbon receptor (AHR). Given their common roles in chemically induced toxicity, we asked whether interleukin 1 (IL1)-like cytokines play a role in acute aspects of the dioxin response. To test this idea, we employed a “triple null” mouse model that lacks the two receptors for the tumor necrosis factors- α and - β and the receptor for the IL1- α and IL1- β cytokines. When triple null mice were treated with dioxin, there was significant attenuation in the levels of serum alanine aminotransferase, signifying reduced hepatocellular damage. Additionally, the triple null mice were protected from dioxin-induced liver inflammation. Loss of receptors for the IL1-like cytokines was not protective for all aspects of dioxin toxicity. Endpoints such as thymic involution, *Cyp1a2* induction, hepatomegaly and hydropic degeneration remain unchanged in this model.

INTRODUCTION:

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) causes a wide spectrum of toxic effects, including hepatocellular damage, thymic involution, teratogenesis and cancer (Pohjanvirta and Tuomisto, 1994). The aryl hydrocarbon receptor (AHR), a member of the PAS superfamily of transcriptional regulators, mediates most, if not all, of these effects (Carver et al., 1998; Poland and Glover, 1980; Schmidt and Bradfield, 1996). The binding of dioxin to the AHR results in translocation of the receptor complex to the nucleus, where the receptor dimerizes with another basic helix loop helix (bHLH) PAS protein known as AHR nuclear translocator (ARNT) (Schmidt and Bradfield, 1996). In the nucleus, the AHR-ARNT heterodimer binds to genomic “dioxin-response elements” (DREs) and upregulates genes encoding a battery of enzymes, including the cytochrome P450-dependent monooxygenases *Cyp1a1*, *Cyp1a2* and *Cyp1b1* (Schmidt and Bradfield, 1996). The induction of these enzymes by the AHR is often referred to as an adaptive response since many AHR agonists such as benzo(a)pyrene both induce this pathway and are metabolized through this response (Gu et al., 2000).

The interleukin 1 (IL1)-like cytokines are soluble factors that are known to participate in chemically-induced liver injury (Schumann and Tiegs, 1999; Yin et al., 1999). These cytokines are commonly thought of as part of the innate immunity mechanism that regulates acute phase proteins under cellular stress (Ramadori and Armbrust, 2001). The IL1-like cytokines that are released under similar physiological and pathological conditions include tumor necrosis factor α (TNF α), tumor necrosis factor β (TNF β), interleukin 1 α (IL1 α) and interleukin 1 β

(IL1 β) (Moshage, 1997; Ramadori and Armbrust, 2001). The TNF α and TNF β cytokines can each bind to 2 known receptors, TNFR1 (p55) and TNFR2 (p75) encoded by the *Tnfrsf1a* and *Tnfrsf1b* loci respectively (Ramadori and Armbrust, 2001). The IL1 α and IL1 β cytokines bind to the type I and the type II receptors encoded by the *Il1r1* and *Il1r2* loci respectively. However, the binding to the type II receptor is not known to initiate signaling and it is widely accepted that this receptor acts as a “decoy” that prevents IL1 binding to the type I receptor (Glaccum et al., 1997). The IL1-like cytokines act in both autocrine and paracrine manners by binding to their respective receptors on target cells resulting in the initiation of various intracellular signaling cascades. An example of this is the upregulation of the NF κ b and the MAP kinase pathways that are induced in acute phase response by both TNF and IL1 (Moshage, 1997; Ramadori and Armbrust, 2001).

The common involvement of IL1-like cytokines in forms of chemically induced liver injury led us to hypothesize that these cytokines are important downstream mediators of certain aspects of dioxin-induced hepatotoxicity. For example, TNF α has been shown to participate in the acute liver injury that occurs in response to concanavalin A, endotoxin and alcohol (Bradham et al., 1998; Diehl, 2000; McClain et al., 1998; Yin et al., 1999). Both TNF α and IL1 α have also been shown to play a role in acetaminophen-induced liver toxicity (Blazka et al., 1996). In consideration of the overlapping patterns of intracellular signaling and gene expression activated by the various IL1 and TNF agonists and receptors, we generated a “triple null” mouse line that is deficient for all known IL1 and TNF signaling. This was accomplished by generating compound mutants lacking all three functional receptors, namely TNFR1, TNFR2, and IL1R1.

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In this paper, we utilize this triple null mouse model to identify those aspects of dioxin-induced toxicity that are dependent on IL1-like cytokine signaling.

MATERIALS AND METHODS:

Animals: Animals were housed in a specific pathogen-free facility on corn cob bedding with food and water *ad libitum* following the rules and regulations established by the University of Wisconsin Animal Care and Use Committee.

*Generation of “triple null” mice lacking *Tnfrsf1a*, *Tnfrsf1b* and *Il1r1*:* We obtained mice that harbor null alleles at the *Tnfrsf1a*, *Tnfrsf1b*, and *Il1r1* loci from the Jackson Laboratory, Bar Harbor, Maine (Erickson et al., 1994; Glaccum et al., 1997; Pfeffer et al., 1993). Although we use the abbreviated nomenclature above, the specific nomenclature for the *Tnfrsf1a*, *Tnfrsf1b*, and *Il1r1* lines is B6.129-*Tnfrsf1a*^{tm1Mak}/J, B6.129S2-*Tnfrsf1b*^{tm1Mwm}/J, and B6.129S7-*Il1r1*^{tm1Imx}/J respectively (Rules for Nomenclature of Mouse and Rat Strains, The Jackson Laboratory Web Site, The Jackson Laboratory, Bar Harbor, MN, URL: <http://www.informatics.jax.org/mgihome//nomen/strains.shtml> [December 2004]). To generate the triple null, *Tnfrsf1a*^{-/-} and *Tnfrsf1b*^{-/-} mice were first interbred to generate a *Tnfrsf1a*^{-/-}/*Tnfrsf1b*^{-/-} double null line. This line was then crossed with an *Il1r1*^{-/-} mouse to generate a *Tnfrsf1a*^{-/-}/*Tnfrsf1b*^{-/-}/*Il1r1*^{-/-} “triple null” mouse line. Mice were genotyped by PCR protocols established for the individual alleles using genomic DNA isolated from tail biopsies (Erickson et al., 1994; Glaccum et al., 1997; Pfeffer et al., 1993). Additionally, RT-PCR was carried out to examine expression from the individual alleles using liver total RNA (RNeasy Midi Kit, Qiagen, Valencia, CA). The primer sets used for the RT-PCR were: *Tnfrsf1a* allele, forward primer OL 5655 (5' – GCTTGTGTCCCAAGGAAAG) and reverse primer OL 5656 (5' – GTCCTGGGGGTTTGTGACAT); *Tnfrsf1b* allele, forward primer OL 5659 (5' –

TCTAGCTCCAGGCACAAGG) and reverse primer OL 5660 (5' – CATTGGGGCTCTTGAAGT); *Il1r1* allele, forward primer OL 5662 (5' – TTGAGGAGGCAGTTTTTCGTT) and reverse primer OL 5663 (5' – TACCACTGGACCTCGGGTAA). Since individual receptor null animals had been crossed to the C57BL/6J (B6) background for at least 5 generations prior to import, the triple null line is over 95% B6 background.

Dioxin hepatotoxicity: At 7-8 weeks of age, male mice were intraperitoneally injected with either 64 µg/kg dioxin or, the same volume of vehicle alone (DMSO). Fourteen days after the dioxin treatment, mice were anesthetized using isoflurane and blood was obtained by cardiac puncture. Liver and thymus weights were then measured and normalized to whole body weights. Medial sections from the left lobe of the livers were fixed in 10% (w/v) buffered formalin for histological examination. Apoptosis was determined by TUNEL (TdT-mediated dUTP nick-end labeling) assay using *In Situ* Cell Death Detection Kit, POD (Roche Applied Science, Indianapolis, IN). The number of TUNEL positive cells was estimated using Stereo Investigator software (MicroBrightfield Inc., Williston, VT). To visualize macrophage/ monocyte populations, immunostaining was performed with rat anti-mouse F4/80 monoclonal antibody (Serotec Inc., Raleigh, NC), biotinylated rabbit anti-rat secondary antibody (Vector Labs, Burlingame, CA) and avidin-biotin conjugated system with NovaRED substrate (Vector Labs, Burlingame, CA) (Martinez-Pomares et al., 1996). The number of inflammatory macrophages was estimated from F4/80 immunostained slides using Stereo Investigator software (MicroBrightfield Inc., Williston, VT). Hematoxylin & Eosin (H&E) stained sections were used to estimate the percentage of cells displaying hydropic degeneration as follows: 0 (absent); 1

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(rare, scattered); 2 (below 25%); 3 (25 – 50%); 4 (50 – 75%); 5 (75 – 100%). Alanine aminotransferase (ALT) activity was determined on sera by the Clinical Pathology Laboratory at the University of Wisconsin, School of Veterinary Medicine in Madison. Statistical analysis was performed using an ANOVA and Tukey's test was used to determine differences with $p < 0.05$.

Northern analysis: *Cyp1a2* expression was determined with Northern analysis using 10 µg total RNA. The CYP1A2 probe (Plasmid 1232) was labeled with ^{32}P dCTP using Rediprime II Random Primer DNA Labeling System (Amersham Biosciences, UK).

RESULTS:

*Generation of mice deficient for *Tnfrsf1a*, *Tnfrsf1b* and *Il1r1*:* To determine if IL1-like cytokines are important in dioxin toxicity, we generated mice lacking the three receptors for the four IL1-like cytokines – IL1 α , IL1 β , TNF α , and TNF β . The triple null was generated by intercrossing mice having individual null alleles until animals homozygous at all the three null loci were obtained. To confirm the presence of all three null alleles, a PCR genotyping approach was performed based on published protocols (Erickson et al., 1994; Glaccum et al., 1997; Pfeffer et al., 1993). To demonstrate that each targeted allele resulted in disruption in corresponding gene expression, RT-PCR was performed on liver mRNA from the triple null and wild-type mice (Figure 1). For *Tnfrsf1a* and *Tnfrsf1b*, we designed primers that amplify the mRNA corresponding to exons 2-6 and exons 1-5 respectively. For *Il1r1*, the mRNA region corresponding to exons 1-4 was amplified. The RT-PCR products for all three loci were positive for the expected sizes in wild-type mice but not in triple null mice (Figure 1). Although mating efficiency is reduced in triple null mice, the adult animals display body weights and liver histology that is similar to their wild-type controls (data not shown). Given that most null alleles are derived from 129-derived ES cells, we genotyped the triple null strain at the *Ahr* locus and confirmed that they possess the high-affinity allele, *Ahr*^{b-1}, commonly found in B6 strain and not the low-affinity allele, *Ahr*^d, commonly found in 129 strain (data not shown) (Poland et al., 1987).

*Hepatomegaly, thymic involution, hepatic hydropic changes, and *Cyp1a2* induction are unchanged in dioxin exposed triple null mice:* Both wild-type and triple null mice exposed to

dioxin at 64 $\mu\text{g}/\text{kg}$ display similar levels of hepatomegaly, thymic involution and hepatic hydropic changes (Figure 2A-2C). The relative liver weights in wild-type mice increased by 30% while the triple null relative liver weight increased 32% in response to dioxin (Figure 2A). Similarly, the wild-type thymus weight decreased 72% and the thymus weight in triple null mice was reduced 69% as a result of dioxin exposure (Figure 2B). Dioxin exposure also caused similar levels of hydropic degeneration in both wild-type and triple null mice at 14 days (Figures 2C). We define hydropic degeneration as vacuoles in the cytoplasm of hepatocytes that meet three criteria. First, they have a ragged perimeter. Second, they are negative for lipid by oil-red-o staining. Third, they are negative for glycogen as determined by PAS staining. A qualitative scoring of H&E sections from all liver sections did not reveal even a subtle difference when comparing control wild-type and triple null or when comparing dioxin-treated wild-type and triple null (Figures 2C and 2D). Induction of the *Cyp1a2* gene product was used as a classical response to dioxin activated AHR signaling. We assessed *Cyp1a2* expression using Northern analysis in each of the four treatment group (Figure 2E). In both wild-type and triple null mice, dioxin treatment resulted in similar levels of induction of the *Cyp1a2* gene.

Triple null mice are protected from dioxin-induced hepatocellular damage: We observed that dioxin exposure led to infiltration of inflammatory cells in the livers of wild-type mice (Figures 3A and 3B). The types of inflammatory cells identified include macrophages, monocytes as well as smaller number of lymphocytes and neutrophils. The density of infiltrating F4/80 positive macrophages and monocytes within the liver increased over 23 fold after 64 $\mu\text{g}/\text{kg}$ dioxin treatment of wild-type mice. When comparing wild-type mice, this difference between dioxin treated and control was significantly different at $p < 0.001$ (Figure 3B). In triple null mice,

dioxin-induced increase in the density of macrophages and monocytes was also observed. Yet this increase was not statistically different from the corresponding control group at $p < 0.05$ (Figure 3B). Importantly, dioxin-treated wild-type mice exhibited about 4-fold greater density of infiltrating macrophages as compared to dioxin-treated triple null mice. This difference was found to be statistically significant at $p < 0.01$.

We also observed that dioxin causes significant hepatocellular damage in wild-type mice, as indicated by an over 21-fold elevation in serum ALT levels (Figure 3C). In contrast, the elevation of serum ALT in triple null mice exposed to dioxin was only about 4-fold of control and the difference was not statistically significant at $p < 0.05$ (Figure 3C).

Dioxin-induced hepatocyte apoptosis is attenuated in triple null mice: In dioxin-exposed wild-type mice, we observed induction of hepatocyte apoptosis in regions corresponding to regions of inflammatory cell infiltration (Figure 4A). Therefore, we used TUNEL staining as an additional measure of dioxin-induced hepatocellular toxicity and an indirect measure of liver inflammation. Dioxin caused 10-fold increase in hepatocyte apoptosis in wild-type mice and this difference was significant at $p < 0.001$ (Figures 4B & 4C). Likewise, the difference between dioxin-treated wild-type mice and dioxin-treated triple null mice was significant at $p < 0.001$. In contrast, only marginal apoptosis was seen in triple null mice treated with dioxin and the difference between control and dioxin treated mice was not significant at $p < 0.05$ (Figure 4C).

DISCUSSION:

To demonstrate a role of IL1-like cytokines in dioxin toxicology, we generated a triple null mouse line that is deficient in the receptors for TNF α , TNF β , IL1 α and IL1 β . Exposure of these mice to dioxin demonstrated that IL1-like cytokines are involved in particular aspects of dioxin toxicity. We observed that dioxin-induced hepatocellular damage as assessed by serum ALT levels, hepatic infiltration by inflammatory cells, and hepatocyte apoptosis were all significantly reduced in triple null animals. Despite the significant protection for these endpoints, the triple null genotype had no influence on dioxin-induced thymic involution, hepatomegaly, *Cyp1a2* induction or, hydropic degeneration. Taken in sum, these data support the idea that some, but not all responses to dioxin are dependent upon signaling by the IL1-like cytokines.

These observations are in keeping with the idea that dioxin toxicity is multi-factorial and that distinct sets of signaling events lead to different toxic endpoints. One example of this idea is the observation that dioxin-induced hepatomegaly occurs normally in triple null mice while other toxic endpoints are reduced. One model to explain this result is that hepatomegaly is a direct consequence of the dioxin-induced P450 enzymes. The coordinated proliferation of smooth endoplasmic reticulum may underlie increased hepatocyte size and concomitant hepatomegaly (Schulte-Hermann, 1974; Turner and Collins, 1983). These data also support a model where dioxin hepatotoxicity is a progressive event, where P450 induction, hepatomegaly and hydropic degeneration represent the earlier stages of acute exposure while hepatocellular damage and inflammatory cell infiltration are sequelae that occur at a later stage. In such a model, hydropic degeneration might represent a crucial state where hepatocyte physiology is significantly

disrupted. We propose that this disruption results in IL1-like cytokine release by resident macrophages, subsequent inflammation and further hepatocellular damage. It follows that removal of the IL1-like signal would halt this progression. Thus dioxin exposure would result in P450 induction, hepatomegaly and hydropic degeneration but not hepatocellular damage and inflammatory cell infiltration.

The IL1-like cytokines, TNF α , TNF β , IL1 α and IL1 β , are soluble factors that are known to participate in various aspects of liver injury. The classification of cytokines as “IL1-like” comes from observations of the “acute phase response” where signaling by the IL1-like cytokines can be distinguished from responses stimulated by the interleukin 6 (IL6)-like cytokines. The signaling of IL1-like cytokines results in the induction of type I acute phase proteins (Ramadori and Armbrust, 2001). The three IL1-like cytokine receptors, TNFR1, TNFR2 and IL1R1, are able to activate both overlapping and distinct intracellular pathways (Moshage, 1997; Ramadori and Armbrust, 2001). Because of this overlap in signaling, we chose to develop an animal model that was null for all three receptors and therefore incapable of mediating any IL1-like response. The primary advantage of this model is its greater sensitivity in detecting IL1-like signaling, since pathway redundancy has been reduced through the generation of a compound null.

Although we have not found this to be reported previously, we observed that the regions of dioxin-induced hepatocyte apoptosis correlated with sites of inflammatory cells in treated livers. This overlap provides evidence that inflammation and apoptosis are related; however, it is unclear which event precedes the other. It is possible that hepatocyte apoptosis is not as clean as previously thought and that it triggers infiltration of inflammatory cells which provide support in

clearing up the debris resulting from cell death (Maher and Gores, 1998). Alternately, the recruitment of inflammatory cells to the liver could lead to hepatocellular stress, leading to hepatocellular apoptosis. It is also possible that the two effects amplify each other, i.e., the presence of inflammatory cells result in cellular death; this, in turn, recruits more inflammatory cells to that site. Further experiments are needed to explain which, if any, of these scenarios occur in dioxin-treated animals.

The rationale for our hypothesis that IL1-like cytokines may be involved in dioxin toxicity came from a broad spectrum of experiments that have been published in the last decade. Foremost is the observation that IL1-like cytokines have been shown to be common mediators of the hepatotoxicity induced by a number of agents including alcohol, acetaminophen, endotoxin and concanavalin A (Blazka et al., 1996; Bradham et al., 1998; Diehl, 2000; McClain et al., 1998; Yin et al., 1999). Secondly, a number of inflammatory cytokines have been linked to dioxin toxicity by other laboratories (Charles and Shiverick, 1997; Fan et al., 1997; Fisher et al., 2004; Moos et al., 1994; Taylor et al., 1992). In this regard, dioxin has been shown to induce TNF α and IL1 β mRNAs, supporting the possibility that the expression of these cytokines are DRE-mediated (Fan et al., 1997). Although direct AHR regulation is a formal possibility, our observations are also consistent with a model where these cytokines respond to dioxin indirectly. One model compatible with our results is that P450 induction, hepatomegaly and hydropic degeneration represent primary effects of dioxin. These events could subsequently lead to the disrupted physiology of hepatocytes and the activation of IL1-like cytokines. The cytokine action could then result in the secondary effects, namely hepatocyte damage and infiltration of inflammatory cells.

Using a triple null mouse model where TNF and IL1 signaling are abrogated, we demonstrated clear roles for IL1-like cytokines in mediating aspects of dioxin-induced hepatotoxicity. In addition to its importance in dioxin toxicity, we expect this mouse model to be very useful in other areas of toxicology. In future studies, we plan to address two important questions. First, which subsets of the IL1-like cytokine pathways are essential for these toxic responses (e.g., is it TNF α alone) and, second what other aspects of dioxin toxicity are mediated by IL1-like cytokines (e.g., tumor promotion).

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FOOTNOTES:

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FIGURE LEGENDS:

Figure 1. All three IL1-like receptors are absent in triple null mice. The RT-PCR was performed for each allele to confirm the loss of all three receptors in the triple null. Lanes 1 and 2 show *Tnfrsf1a* mRNA amplification in wild-type and triple null mice respectively. Lanes 3 and 4 show *Tnfrsf1b* mRNA amplification in wild-type and triple null mice respectively. Lanes 5 and 6 show *Il1r1* mRNA amplification in wild-type and triple null mice respectively.

Figure 2. Dioxin induces hepatomegaly, thymic involution, and *Cyp1a2* induction in both wild-type and triple null mice. Male mice (6-8 weeks old) were injected intraperitoneally with either 64 µg/kg dioxin dissolved in DMSO (vehicle) or an equal volume of DMSO alone. Fourteen days after dioxin treatment, liver and thymus weights were measured and normalized to whole body weights (Figs. 2A and 2B). Medial section from the left lobe of the liver was fixed in 10% formalin, sections prepared, and stained with Hematoxylin and Eosin (H&E) to assess the overall liver damage (Fig. 2C). The H&E stained slides were then scored for hydropic degeneration (Fig. 2D). Total RNA from the liver was used to perform Northern analysis to detect the *Cyp1a2* mRNA (Fig. 2E). *Scale*, 100 µm. *White bars*, vehicle treated animals; *gray bars*, dioxin treated animals. *Error bars*, S.E.M. Those groups not sharing a superscript letter differ significantly at $p < 0.001$. The number of animals in each group is at least 4.

Figure 3. Triple null mice are protected from dioxin-induced hepatocellular damage. Male mice (6-8 weeks old) were injected intraperitoneally with either 64 µg/kg dioxin dissolved in DMSO or an equal volume of DMSO alone. After 14 days, medial section from the left lobe of

the liver was fixed in 10% formalin, sections prepared, and stained with F4/80 antibody to count the number of macrophages and monocytes within inflammatory cell clusters (Figs. 3A). The extent of dioxin-induced inflammatory cell infiltration in the triple null is reduced as compared to wild-type mice (Fig. 3B). Blood sera were also obtained from mice for measurement of ALT levels (Fig. 3C). In wild-type mice, dioxin causes significant ALT induction whereas this induction is not significant in triple null mice. *Scale*, 100 μ m. *White bars*, vehicle treated animals; *gray bars*, dioxin treated animals. *Error bars*, S.E.M. Those groups not sharing a superscript letter differ significantly at $p < 0.01$. The number of animals in each group is at least 4.

Figure 4. Dioxin-induced hepatocyte apoptosis is reduced in triple null mice. Male mice (greater than 6 weeks old) were injected intraperitoneally with either 64 μ g/kg dioxin dissolved in DMSO (vehicle) or an equal volume of DMSO alone. After 14 days, medial sections from the left lobe of the dioxin-treated livers from wild-type mice were fixed in 10% formalin, sections prepared, and stained with H&E (Fig. 4A). The TUNEL assay was performed on adjacent liver sections for assessment of hepatocyte apoptosis. Comparison between H&E and TUNEL stained sections in dioxin-treated livers from wild-type mice reveal a high degree of correlation between the sites of inflammatory cells (arrowheads) and hepatocyte apoptosis (Fig. 4A). TUNEL staining was also performed on control and dioxin-treated livers from both wild-type and triple null mice (Fig. 4B). Significant hepatocyte apoptosis is seen after dioxin treatment in wild-type mice but not in triple null mice. The TUNEL-positive cells were estimated using the Stereo Investigator software. In control mice, dioxin induces significant apoptosis whereas this induction is absent in triple null mice (Fig. 4C). *Scale*, 100 μ m. *White bars*, vehicle treated

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animals; *gray bars*, dioxin treated animals. *Error bars*, S.E.M. Those groups not sharing a superscript letter differ significantly at $p < 0.001$. The number of animals in each group is at least 3.

Figure 1:

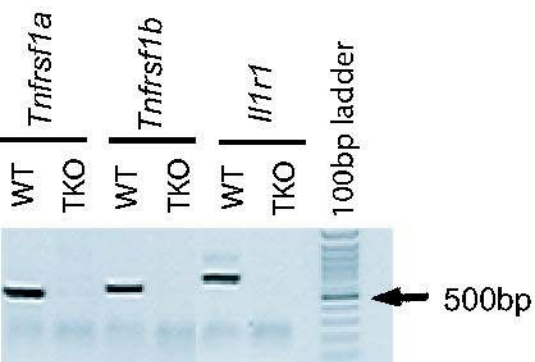


Figure 2:

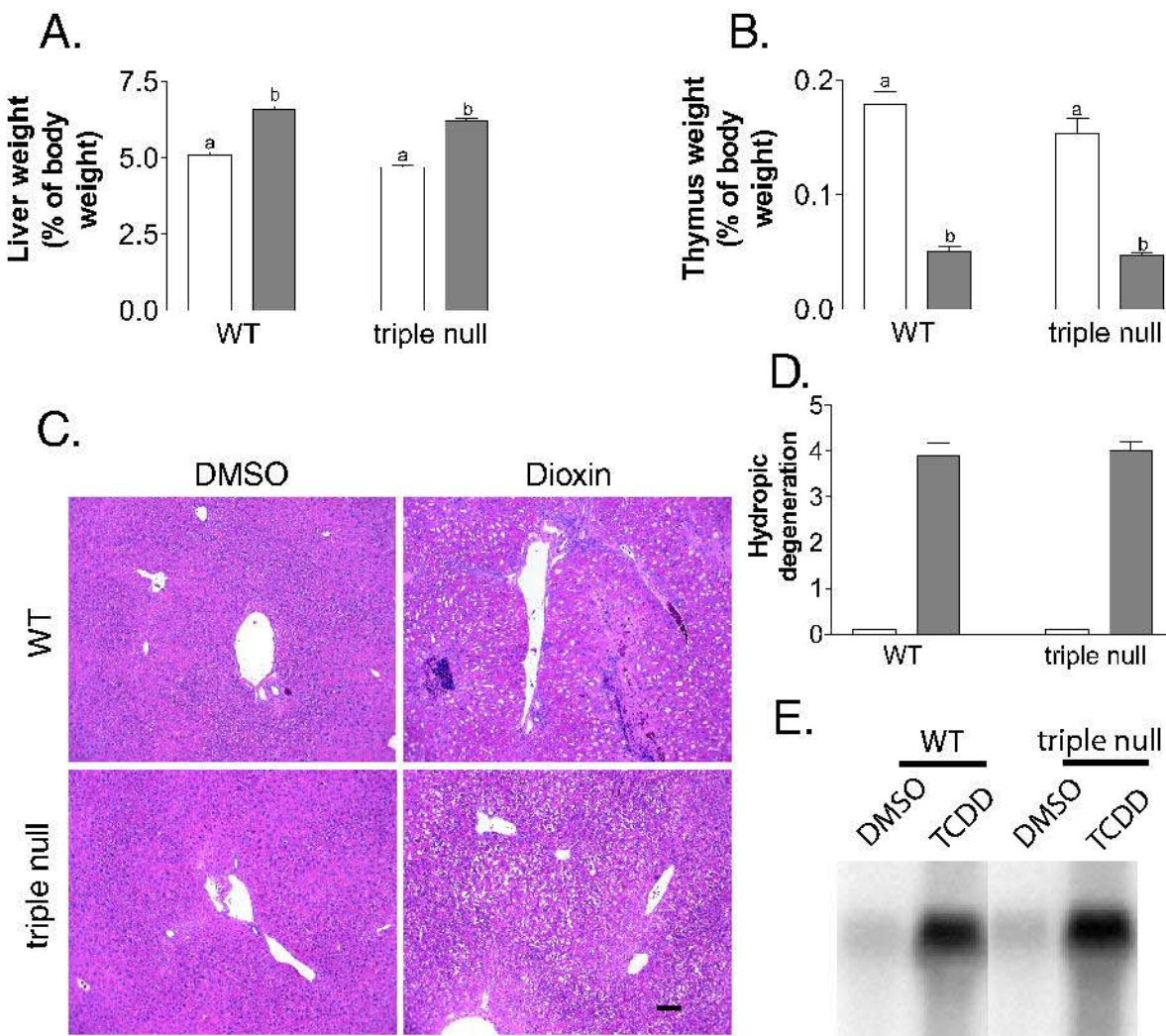


Figure 3:

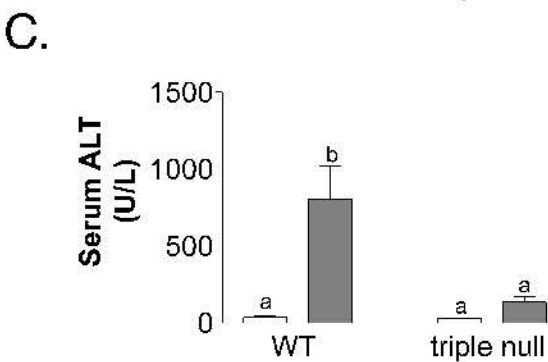
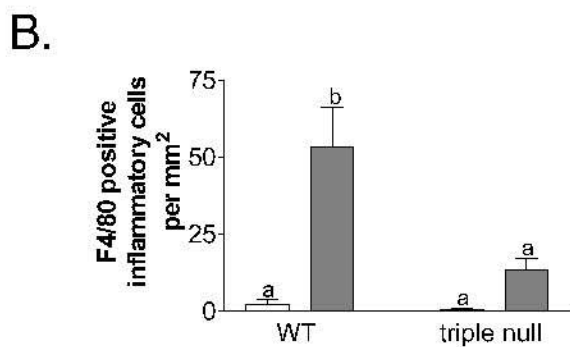
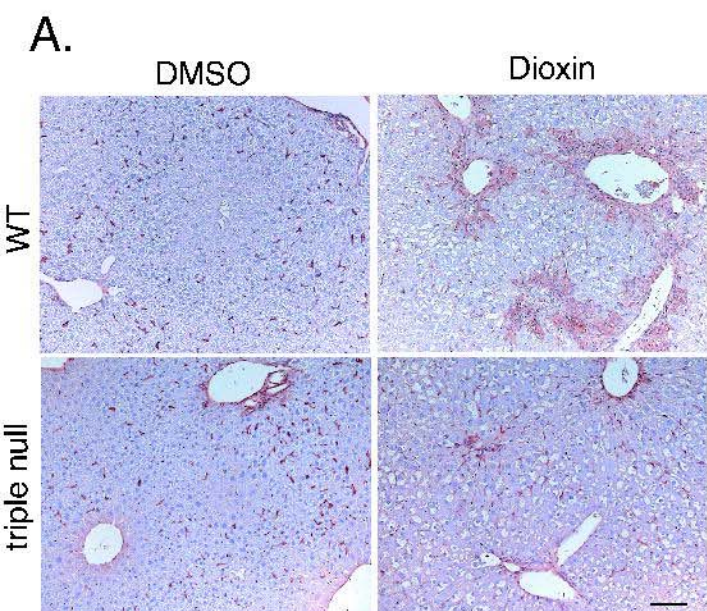


Figure 4:

