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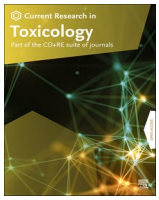

Developmental exposure to thyroid disrupting chemical mixtures alters metamorphosis and post-metamorphic thymocyte differentiation

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ABSTRACT

While there is some evidence to suggest that disruption of the thyroid hormone (TH)-axis during perinatal development may weaken T cell immunity later in life, data are currently lacking on whether environmentally relevant thyroid disrupting chemicals (TDCs) can induce similar outcomes.

To fill this gap in knowledge, *X. laevis* tadpoles were exposed to an environmentally relevant mixture of TDCs, either during early tadpole development, or immediately before and during metamorphosis, to assess T cell differentiation and anti-viral immune response against FV3 infection after metamorphosis. Extending our previous study showing a delay in metamorphosis completion, here we report that TDC exposure prior to metamorphosis reduced the frequency of surface MHC-II + splenic lymphocytes and weakened some aspects of the anti-viral immune response. TDC exposure during metamorphosis slowed post-metamorphic migration of the thymus reduced the renewal of cortical thymocytes and splenic CD8 + T cells. The results indicate that TDC exposure during perinatal development may perturb the formation of T cell immunity later in life.

Introduction

Endocrine disrupting chemicals (EDCs) are well known to induce potent immune alteration when exposure occurs during sensitive windows of development, even long after EDC exposure has ended. EDCs are often found in mixtures, leading to additional challenges in studying their health impacts (Carvalho et al., 2014). Furthermore, data are lacking on whether EDC exposure can perturb immune cell differentiation and development (Gore et al., 2015). Disruption of thyroid hormones (TH) by TH synthesis inhibitors have been shown to affect T cell development during in-utero development in mammals, but the same TH depletion typically do not affect T development in neonates after birth (Albornoz et al., 2013). Early life establishment of the T cell receptor (TCR) repertoire is critical for supporting clonal diversity of T cells later in life that will permit to combat the high diversity of pathogens (Thome et al., 2016). Therefore, it is likely that early life thymocyte differentiation is susceptible to reprogramming by environmental chemicals, particularly for thyroid disrupting chemicals (TDCs) that alter TH.

One possible way that TDCs may impact immune cell differentiation is by altering the TH dependent remodeling of the thymus. In amphibian tadpoles, the immune system is reliant on innate immunity with very few conventional T cells. During metamorphosis the immune system is remodeled, leading to higher T cell output from the thymus in adulthood and a more robust adaptive immune system (Turpen & Smith, 1989). During metamorphosis, high TH and glucocorticoid (GC) levels induce extensive apoptosis of thymocytes, and renewed thymocyte differentiation occurs from lymphopoietic progenitors migrating in the thymus within a few weeks after the metamorphic completion (Rollins-Smith, Blair, & Davis, 1992). Amphibians have long been used to model the TH-axis endpoints of environmental chemicals, while the high degree of evolutionary conservation of their immune system with that of mammals has been underutilized. In mammals, which lack metamorphosis, there is no strict remodeling of the thymus. However, mammals rely on maternal immunity during uterine development and do not need as much immune output from lymphopoietic tissues (Yang, Zheng, & Jin, 2019). In both amphibians and mammals, the thymus undergoes involution and shrinks at adulthood, which is characterized by conversion of lymphopoietic tissue to adipose tissue and a decreased thymic output (Cowan, Takahama, Bhandoola, & Ohigashi, 2020). The stromal cells of the thymus support and trigger differentiation of thymocytes into mature T cells, and the gene expression of the stromal TECs may change

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over the course of development (Brunk et al., 2017). Due to the overall similarity of thymocyte development between amphibians and mammals, *X. laevis* is a powerful model for determining the long-term immune effects of chemical exposures.

Establishment of the TCR repertoire is required for primary T cell responses to viral infections and the formation of immunological memory to prevent reinfection (Marshall, Warrington, Watson, & Kim, 2018). The *X. laevis* model has previously been used to evaluate acute effects of TDC mixture exposure on T cell differentiation, as well as long term effects of EDC mixtures on anti-viral immunity (McGuire, Lawrence, & Robert, 2021), (Robert, McGuire, Nagel, Lawrence, & Andino, 2019). However, the developmental effects of TDC mixture exposure have not yet been determined in *X. leavis*. TDCs are increasingly studied in the context of other organ systems, particularly for neurodevelopment, while emerging TDCs such as PFASs and PCBs are increasingly tested. Many TDCs are used in unconventional oil and gas extraction (UOG), with little to no government regulation for these chemicals (Gross et al., 2013).

To study the ways in which mixtures of environmentally relevant TDCs can alter the metamorphic differentiation of immunity in amphibians, four representative UOG chemicals were chosen. These TDC contaminants are consistently found together in UOG contaminated sites across the United States. We have previously showed that exposure to a mixture of these 4 chemicals at doses found in the environment can delay metamorphosis and induce hypertrophy-like pathology in the thyroid gland as well as short term perturbations of the TH-axis and thymocyte differentiation (McGuire et al., 2021). These chemicals are naphthalene, ethylene glycol, ethyoxylated octylphenol, and ethoxylated nonylphenol. Each of these four chemicals has prior evidence of TH disruption (Table S1). We exposed tadpoles to a mixture these 4 chemicals at equimass doses of 0, 0.1, and 1 μg/L for three weeks. These doses were chosen because they are within environmentally measured reference ranges for these chemicals and represent a realistic range of ecological exposure (Table S1). The objectives of this study were to leverage the *X. laevis* model to study whether mixtures of TDCs associated with UOG can perturb metamorphic thymocyte renewal, and long-term T cell maturity and response during viral infections.

Materials and methods

Animal Husbandry: All animals were obtained from the *X. laevis* Research Resource for Immunology at the University of Rochester (https://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm). All animal experiments were carefully handled with the prior approval and under the University of Rochester Committee on Animal Resources regulations (approval number 100577/2003–151).

Chemical mixture: To produce the 1 μg/L equimass dose of the mixture, the relative weights of all chemicals were equal to each other, for a total of 4 μg/L chemical substances. The chemicals used in these experiments were DMSO (CAS 67–68-5; 99.9 % purity, VWR Radnor PA, USA), ethylene glycol (CAS 107–21-1, anhydrous, liquid, 99.8 % purity, Sigma Aldrich, St. Louis, MO, USA), Napthalene (CAS 91–20-3, crystals, 99 % purity, Sigma Aldrich, St. Louis, MO, USA), ethoxylated nonylphenol (CAS, 84133–50-6, 70 % purity in H20, Sigma Aldrich, St. Louis, MO, USA), and ethoxylated octylphenol (CAS 9002–93-1, liquid, 99 % purity, Sigma Aldrich, St. Louis MO). All chemical stocks were prepared in the DMSO vehicle to ensure solubility in water.

Exposures: For all treatments, pre-metamorphic outbred *X. laevis* tadpoles raised at 21–23 °C that were two weeks old (Niewkoop & Faber stage 51–52, see (De Robertis, 1999)) were treated with water containing DMSO vehicle control (0.01 % DMSO), an equimass mixture of 4 chemicals (Table S1.1) at 0.1, 1.0 μg/L for 3 weeks. Two types of exposure were performed:

(A) In a first set of 2 experiments (Figs. 2-4), 10 tadpoles for each treatment were exposed prior to metamorphosis to determine the long term effect on immune responses at maturity (3 months after metamorphosis). Solutions were refreshed by a complete water change every 7 days in the 3-week duration experiments. Animals were then transferred and raised at 21–23 °C in clean water for 6 months until after metamorphosis completion. At least 3 animals from each treatment group were tested.

(B) A separate set of experiments (Figs. 5-7) was designed to assess the acute effects of TDCs on immune remodeling at the onset of metamorphosis. Three tanks of 4 tadpoles for each treatment (DMSO control, 0.1 and 1.0 ug/ml TDCs) were exposed for 3 weeks at developmental stage 60, which is the climax of metamorphosis. All these animals were then raised at 21–23 °C in clean water until metamorphosis completion (stage 66) when the tail is no longer visible. Effect on T cell development was examined at 7, 14 and 21 days post-metamorphosis (post stage 66) by pooling organs of one individual from each of the 3 tanks (4 samples of 3 pooled tadpoles) used per treatment.

Thymus Imaging: At 21 days post-metamorphosis, 5 froglets per group were photographed Nikon E200 Microscope to measure the position of the thymus. Identical 10x magnification and resolution were used for each image. In ImageJ software (Ver 1.3, National Institutes of Health), thymus-arm distance was measured as the shortest line connecting the thymic tissue (visible through the froglet's skin) with the posterior corner of the arm. Thymus-arm distance was recorded and compared in terms of pixel length.

FV3 Infection: Baby hamster kidney cells (BHK-21, ATCC No. CCL-10) were maintained in DMEM (Invitrogen) containing 10 % fetal bovine serum (Invitrogen), streptomycin (100 μg/ml), and penicillin (100 U/ml) with 5 % CO2 at 37 °C, then 30 °C for infection. FV3 was grown using a single passage through BHK-21 cells and was subsequently purified by ultracentrifugation on a 30 % sucrose cushion. Adult frogs were infected by i.p. injection of 2×10^6 PFU in 100 μL of APBS (Edholm, 2018). Uninfected control animals were mock-infected with an equivalent volume of amphibian APBS. A subset of 3 developmentally (3 replicates) exposed adult frogs (6 months old) per group was injected with the thymidine analog EdU (5-ethynyl-2′-deoxyuridine; Catalog: A10044, Invitrogen, Waltham MA, USA) via i.p. injection approximately 18 h prior to tissue collection for flow cytometry. Frogs were injected 2.5 μg of EdU per 1 g of bodyweight. Three, six- or nine-days post-infection (dpi), animals were euthanized using 0.1 g/l tricaine

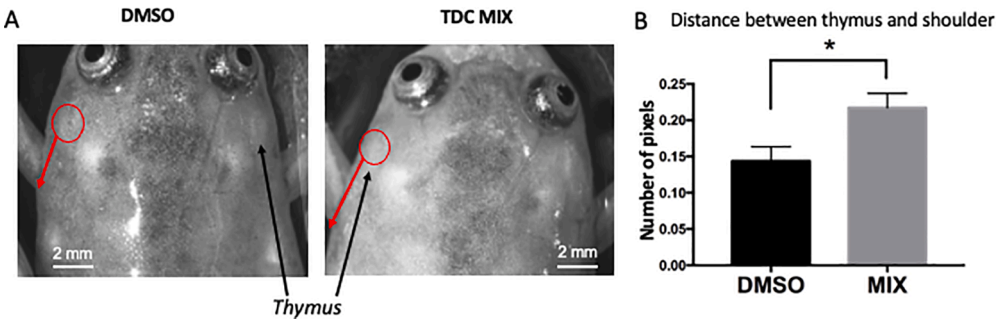

Fig. 1. Quantification of post-metamorphic thymus migration. Stage 60 *X. laevis* tadpoles were exposed to 1 μg/L of the mixture for 21 days. 21 days after the exposure (roughly 28 days post metamorphosis), froglets were imaged for the relative location of their thymic tissue (**A**). The distance between the thymus and the posterior end of the arm connecting to the froglet's shoulder were quantified by ImageJ using number of pixels (**B**). N = 5 for each treatment and * denotes statistical significance using student's *t*-test, error bars denote SEM.

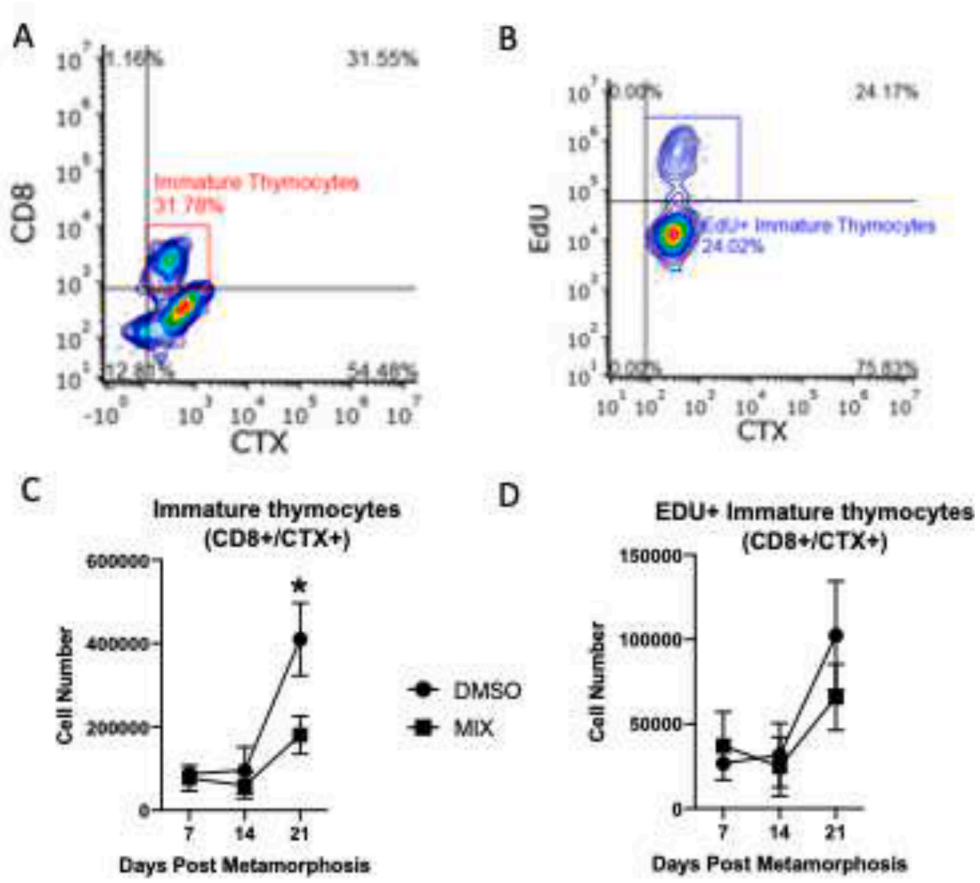

Fig. 2. Quantification of immature cortical thymocytes. Stage 60 *X. laevis* metamorphic tadpoles were exposed to 1 μg/L of the mixture for 21 days. At days 7, 14 and 21 post-metamorphosis, froglet thymocytes were evaluated for CD8, CTX (cortical thymocyte-specific antigen of *Xenopus*) and EdU (5-ethynyl-2′-deoxyuridine) fluorescence via flow cytometry. Representative gating for the frequency of CD8 + and CTX+ (**A**) and proliferative EdU + CD8+/CTX + thymocytes (**B**) are shown. The CD8+/CTX + thymocytes were then quantified for total cell number (**C**) and total number of proliferative EdU + CD8+/CTX + thymocytes (**D**). N = 4 independent samples of 3 pooled tadpoles, * denotes statistical significance using 2-way ANOVA and Tukey's post-test.

methanesulfonate buffered with bicarbonate prior to dissection and extraction immune cells for flow cytometry or nucleic acids from tissues.

Real Time PCR: Tadpole spleen and kidney tissues were harvested, and total RNA was extracted from the tadpole's tissues using TRIzol reagent, following the manufacturer's protocol (Invitrogen, Waltham MA, USA). A total of 500 ng of RNA from each sample was used to synthesize complementary DNA (cDNA) by the Moloney Monkey Leukemia Virus (M−MLV) reverse transcriptase (Invitrogen, Waltham, MA, USA) with oligo(dT) primers (Invitrogen, Waltham, MA, USA). For reverse transcription (RT)-PCR, 125 ng of cDNA was used to determine the expression levels of genes of interest by ΔΔCT value using an ABI 7300 Real-Time PCR System and PerfeCTa SYBR Green FastMix (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Succinctly, for a final reaction of 20 μL, 2.5 μL of cDNA template is added to a mix of 2.5 μL of each primer, 7.5 μL of the master mix buffer and 5 μL of SYBR Green. Primers used are indicated in Table S1.2. Relative gene expression levels were assessed using the by ΔΔCT method (Livak & Schmittgen, 2001). Briefly, expression levels were normalized to an endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), then further normalized against the control group. GAPDH is a useful housekeeping gene due to its consistent and high expression levels between the same tissues of different individuals (Barber, Harmer, Coleman, & Clark, 2005), which did not significantly differ between groups in our experiments. All the primers were validated prior to use by gradient PCR as described in prior literature (Navarro, Serrano-Heras, Castaño, & Solera, 2015). FV3 genome copy numbers were quantified by qPCR using a serially diluted standard curve of a FV3 DNA Pol II PCR fragment cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The qPCR ran for 45 cycles at 65°Celsius, using an NTC (no template control) and clean water control as negative controls to ensure no contamination in our qPCR conditions. Two technical replicates per sample were used (Navarro et al., 2015).

Flow Cytometry: Flow cytometry analysis was conducted with freshly isolated froglet (N = 4 per timepoint per dose) and adult frog immune tissues (N = 3 per dose). To isolate splenocytes and thymocytes, the whole spleen and thymus were disrupted using a 100 μm pore size mesh and rinsed with amphibian phosphate buffered saline (APBS), a more dilute form of PBS suited to the lower tonicity required for amphibian cells. Cells were then suspended in APBS containing 1 % BSA and 0.05 % sodium azide and incubated with monoclonal antibodies. All Xenopus-specific monoclonal antibodies (mAbs) that are not commercially available were produced in-house by the URMC *X. laevis* Research Resource for Immunology (reviewed in Robert et al., 2003; Robert and Ohta, 2009). Each mAb was generated using hybridomas cultures, then tested and titrated for use in flow cytometry using *X. laevis* splenocytes (1:100 dilution). Dual staining of monoclonal Abs was enabled by biotin conjugation of the second Ab used in each cellular stain (Even-Desrumeaux & Chames, 2012) (Edholm, 2018). Monoclonal Abs were then labeled with secondary Abs (Goat anti-mouse FITC (Catalog: A32723) and PE conjugated with streptavidin (Catalog: 12–4317-87), both from Thermo Fisher Scientific, Waltham, MA, USA) at a 1:100 dilution. Following extracellular staining, cells were fixed with 2 % paraformaldehyde (Catalog: 101176–014, VWR) and permeabilized with 0.5 % Triton-100X (Catalog:0694 VWR, Radnor PA, USA) for intracellular staining of thymidine analog EdU incorporated in proliferating cells. Permeabilized cells were incubated with a reaction mix consisting of 1 μL Cy5 Sulfo-Azide Dye (Catalog: A3330, Lumiprobe, Hunt Valley, MD USA), 50 ul of Sodium Ascorbate, 10ul Copper Sulfate, and 440ul of Tris Buffered Saline (100 mM 7.6 pH). Each sample was then washed with APBS containing 1 % BSA and 0.05 % sodium azide twice to reduce background fluorescence.

Cells were evaluated using an LSR II Flow Cytometer (BD Biosciences) with compensation parameters. Fifty thousand events per

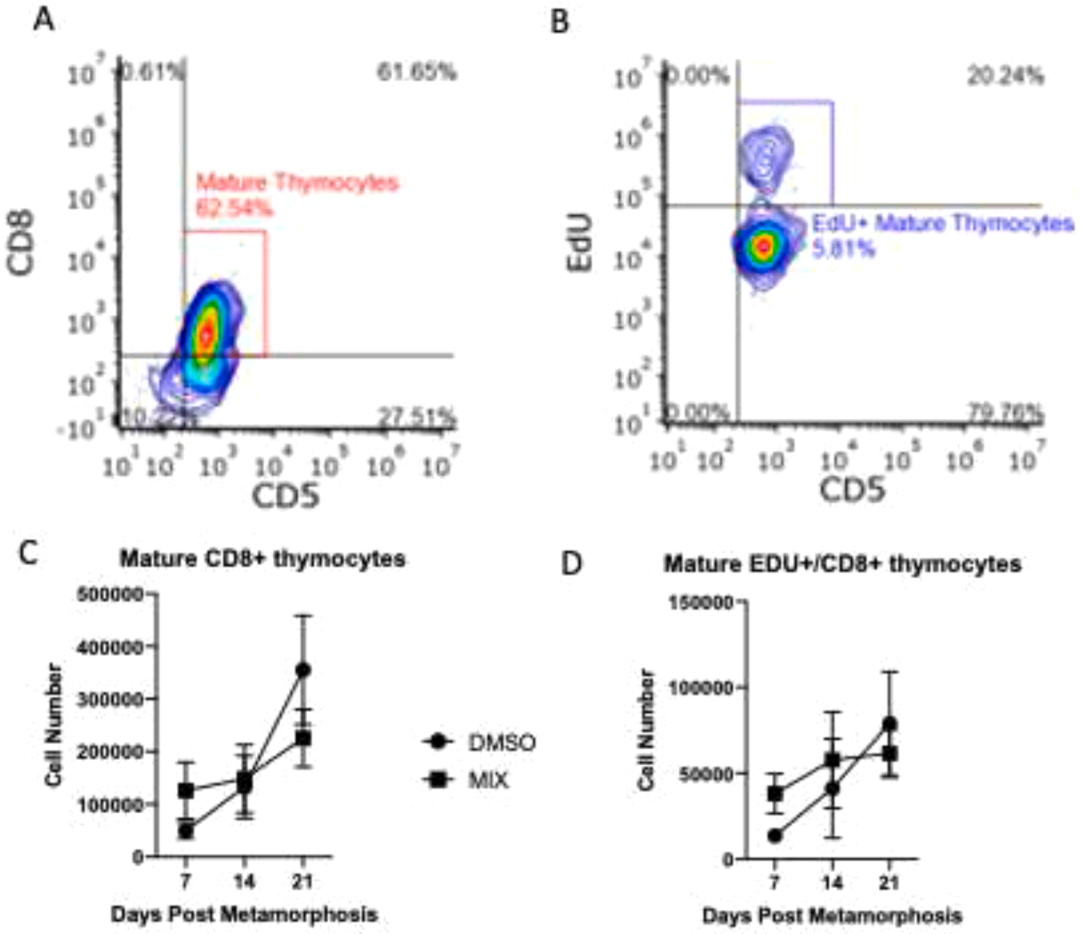

Fig. 3. Quantification of mature medullar thymocytes. Stage 60 *X. laevis* tadpoles were exposed to 1 μg/L of the mixture for 21 days. At days 7, 14 and 21 post-metamorphosis, froglet thymocytes were evaluated for CD8, CD5 and EdU fluorescence via flow cytometry. Representative gating for the frequency of CD8 + and CD5+ (**A**) and proliferative EdU + CD8+/CTX + thymocytes (**B**) are shown. The CD8+/CD5 + thymocytes were then quantified for total cell number (**D**) and total number of proliferative EdU + CD8+/CD5 + thymocytes (**D**). N = 4 per group (treatment and timepoints are separate groups), * denotes statistical significance using 2-way ANOVA and Tukey's post-test.

sample were collected to ensure adequate number of thymocytes were analyzed relative to other cell types such as thymic epithelial cells or red blood cells. To direct the identification of distinct thymocyte populations, gating controls consisting of unstained samples, secondary antibody only controls, and single stained controls. Flow cytometry data were analyzed using FCS Express (Version 7, BD Biosciences).

Statistical Methods: For all experiments, statistical analyses were performed in Graphpad Prism 7 according to the experimental design of each experiment. Normality was tested for all experiments, and proportional data (% frequencies from flow cytometry) was transformed with a logit transformation to ensure normality for analysis via ANOVA. For experiments with two groups a Student's *t*-test was used, while one-way ANOVA and Tukey's post-hoc test was used for experiments with more than two groups. Two-way ANOVA and Tukey's post test were used to evaluate effects over different time points in some experiments. Tukey's multiple comparison test was used to correct for p-values for multiple comparisons.

Results

Exposure to the TDC mixture perturbates the thymus migration during metamorphosis

During metamorphosis, the two lobes of the thymus migrate from a position adjacent to the eyes posteriorly toward the shoulder as a critical part of TH dependent post-metamorphic development, which can potentially be sensitive to TDC (Rollins-Smith et al., 1992). Thus, distance between the thymic lobe and the posterior of the arm was measured and compared, revealing that TDC exposed froglets had significantly longer thymus-arm distance compared to DMSO exposed control frogs, suggesting a delay in migration of these organs (Fig. 1). We did not observe other obvious differences in body shape or size of treated animals compared to untreated controls, and the size of the thymus was not markedly different either.

Exposure to the TDC mixture during metamorphosis alters thymocyte development post-metamorphosis

Within the thymus, thymocytes undergo massive apoptosis during metamorphosis and new thymocytes are replenished following metamorphosis to establish the adult type of splenic T cell repertoire. In a first set of experiment (Exposure A, Materials and Methods), we assessed the acute effects of TDCs on immune remodeling at the onset of metamorphosis. We monitored the replenishment of lymphoid progenitor cells in the thymus and the efficiency of T cell development post-metamorphosis by flow cytometry analysis of thymocytes and splenic lymphocytes every 7 days after the metamorphosis completion (stage 66) following exposure to the TDC mixture during the metamorphic climax (stage 60). Exposure to the TDC mixture had no significant effects on thymocytes nor splenocytes at days 7 and 14 post-metamorphosis

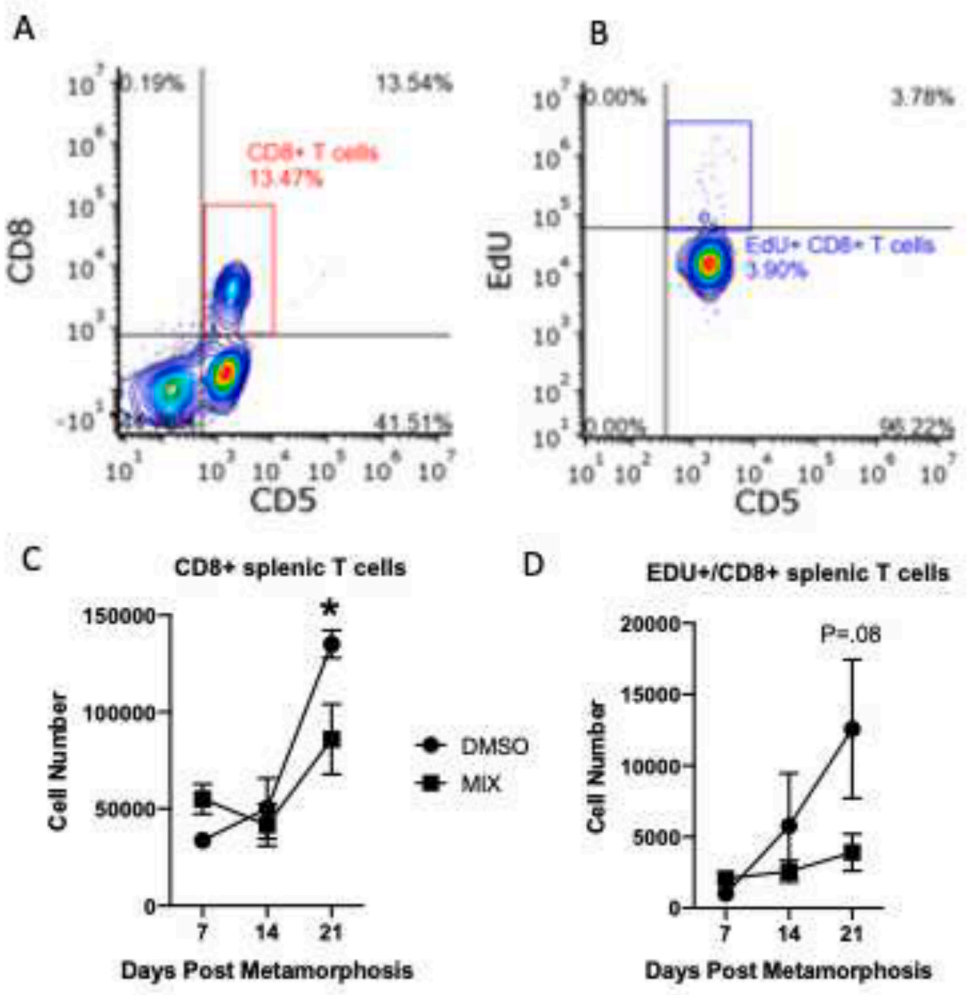

Fig. 4. Quantification of splenic CD8 + T cells. Stage 60 *X. laevis* tadpoles were exposed to 1 μg/L of the mixture for 21 days. At days 7, 14 and 21 post-metamorphosis, froglet thymocytes were evaluated for CD8, CD5 and EdU fluorescence via flow cytometry. Representative gating for the frequency of CD8 + and CD5+ (**A**) and proliferative EdU + CD8+/CTX + splenocytes (**B**) are shown. The CD8+/CD5 + thymocytes were then quantified for total cell number (**C**) and total number of proliferative EdU + CD8+/CD5 + thymocytes (**D**). N = 4 per group (treatment and timepoints are separate groups). * Denotes statistical significance using 2-way ANOVA and Tukey's post-test.

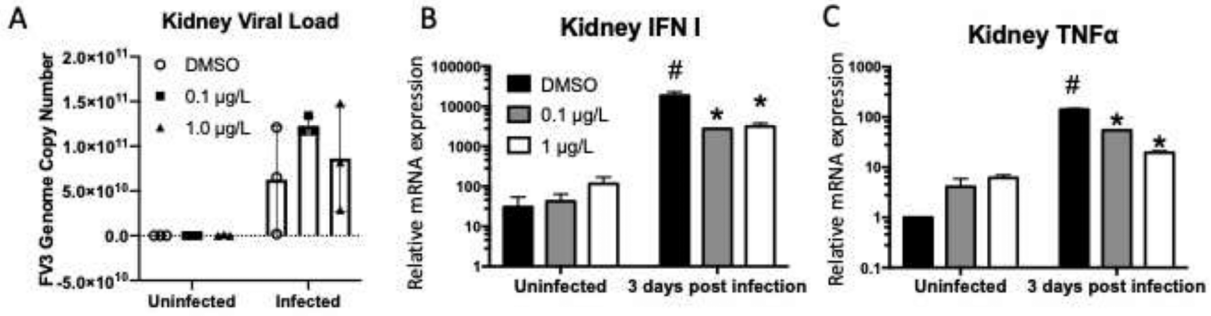

Fig. 5. Anti-viral response against FV3 challenge. Stage 52 tadpoles were exposed to 0.1 and 1 μg/L of the mixture for 3 weeks and then maintained in clean water for 6 months to reach full maturity. Frogs were then infected with FV3, and kidneys were harvested 3 days post infection. (**A**): Quantification of FV3 genome copy number in uninfected and infected animals. (**B**) Relative mRNA expression of anti-viral cytokines Type I IFN and TNFα. N = 3 per group. * Denotes statistical significance using one-way ANOVA and Tukey's post-test among infected groups. # Denotes statistical significance using one-way ANOVA and Tukey's post-test between uninfected DMSO treated control and DMSO -treated FV3 infected animals.

(Figs. 2, 3, and 4). However, at day 21 post-metamorphosis, there was a significant decrease in the number of immature cortical thymocytes (Fig. 2) and the number of mature CD8 + splenic T cells (Fig. 4). To assess thymocyte's proliferation, we used the thymidine analog 5-Ethynyl-2′-deoxyuridine (EdU) that can be incorporated into replicating DNA and is detected by a fluorescent assay. While there were no significant differences in the number of proliferative EdU + thymocytes, there was a non-significant decrease in the number of proliferative EdU+/CD8 + splenic T cells. Overall, there was a lower frequency of proliferative cells in the spleen relative to the thymus, consistent with expected patterns of steady state lymphocyte proliferation in both tissues.

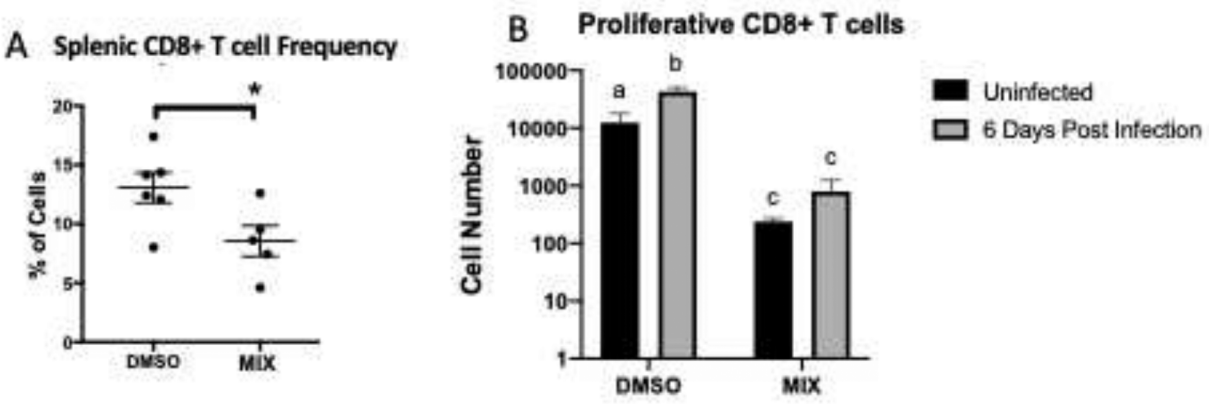

Fig. 6. CD8 + T cell proliferation during FV3 infection. Stage 52 tadpoles were exposed to 1 μg/L of the mixture for 3 weeks and then maintained in clean water for 6 months to reach full maturity. Frogs were then infected with FV3, and splenocytes were harvested and prepared for flow cytometry 6 days post infection. Data presented includes quantification of frequency of CD8 + T cells at steady state (**A**), and proliferative CD8 + T cells during FV3 infection (**B**). N = 3 per group, * denotes statistical significance using one-way ANOVA (with logit transformation of % data) and Tukey's post test for (A) and 2-way ANOVA and Tukey's post-test for (B). Statistical significance in B is denoted by different lowercase letters, where means that differ significantly have different letters from one another, while means that do not significantly differ have the same letter.

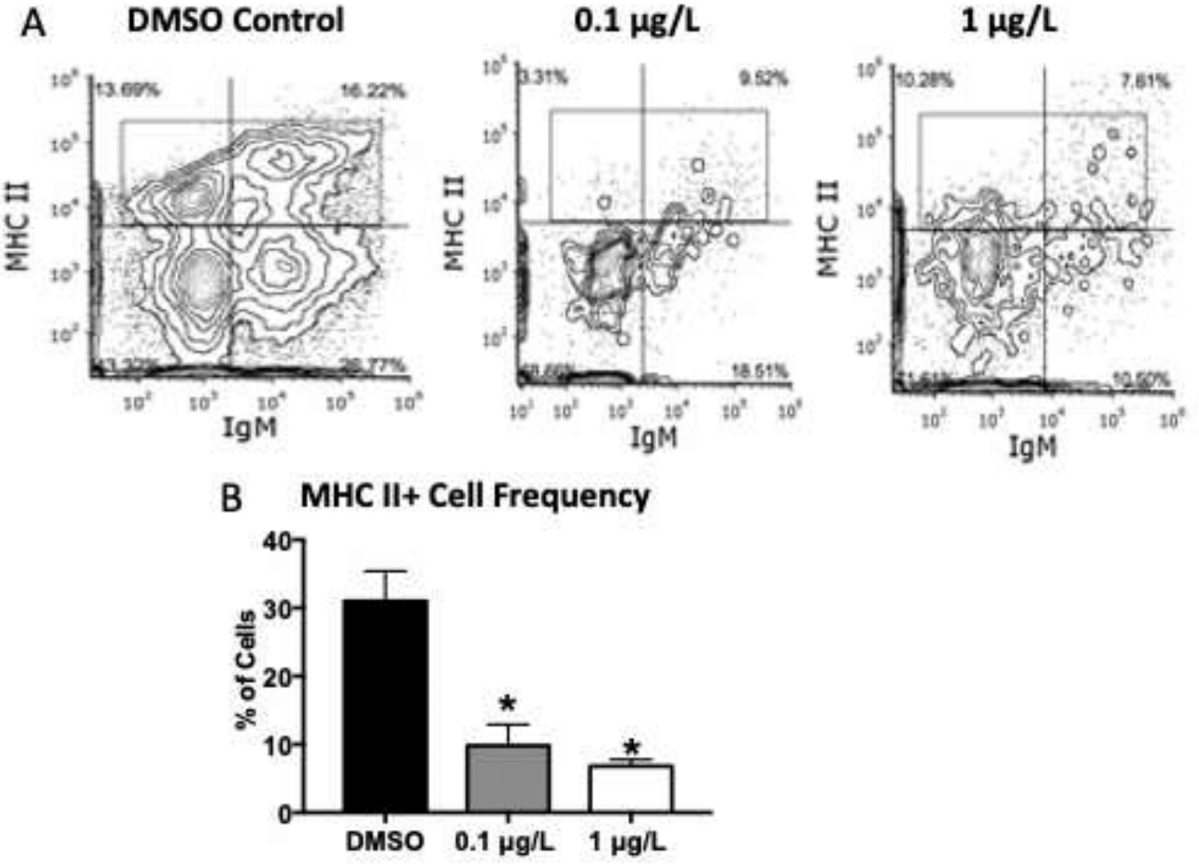

Fig. 7. Quantification of MHC-II + splenic lymphocytes. Stage 52 tadpoles were exposed to 0.1 and 1 μg/L of the mixture for 3 weeks and then maintained in clean water for 6 months to reach full maturity. Splenocytes were then prepared for flow cytometry and gated lymphocytes evaluated for expression of MHC-II and IgM. Representative gating is displayed for each group (**A**), with quantification of the frequency of total MHCII + lymphocytes (**B**). N = 3 per group, * denotes statistical significance using one-way ANOVA (with logit transformation of % data) and Tukey's post-test.

Exposure to the TDC mixture prior to metamorphosis weaken the anti-viral immune response against FV3 pathogens and reduces the frequency of MHC-II + lymphocytes

A second set of experiments (exposure B, Materials and Methods) was designed to determine whether developmental exposure to the TDC mixture induces long lasting effect on T cell development and anti-viral immunity later in life. Developmentally exposed *X. laevis* tadpoles were infected with FV3 six months after metamorphosis. No significant difference in FV3 viral loads were detected by qPCR on kidney genomic

DNA (Fig. 5**A**). Relative gene expression analysis by qPCR in kidney revealed that expression of genes encoding anti-viral cytokines type I IFN and TNF-alpha was significantly reduced in frogs that were developmentally exposed to TDC (Fig. 5**B**). Flow cytometry analysis of splenocytes showed significantly fewer CD8 + T cells in frogs developmentally exposed to the TDC mixture (Fig. 6**A**). When evaluating CD8 + T cell expansion, a critical part of the T cell response to viral infections, there was strong CD8 + T cell proliferation in DMSO exposed frogs, whereas TDC mixture exposed frogs did not have a significant increase in CD8 + T cell proliferation (Fig. 6**B**). These results indicate that developmental exposure to the TDC mixture persistently weakens the host anti-viral response to FV3 infection.

Unlike tadpole lymphocytes that are surface MHC-II negative (Du Pasquier & Flajnik, 1990), splenic lymphocytes will express MHC-II at the cell surface as a marker of maturity after metamorphosis, which is known to be strictly dependent on TH signals during metamorphosis (Rollins-Smith, Davis, & Blair, 1993). It was, therefore, of interest to determine whether developmental exposure to the TDC mixture had any impact on the occurrence of class II + lymphocytes. Six months after metamorphosis completion, splenocytes were harvested and gated lymphocyte were evaluated for MHC-II and IgM surface expression by Flow Cytometry. IgM surface staining, a marker of mature B cell that express high level of MHC-II (Fig. 7A) was used as positive control. A strong and significant decrease in the frequency of surface MHC-II + lymphocyte was found upon developmental exposure to the TDC mixture, in both B cells (expressing surface IgM and high level of MHC-II) and T cells (expressing lower level of MHC-II but not IgM) (Fig. 7).

Discussion

Metamorphosis is a critical event in the amphibian life cycle, conferring advantageous survival and fitness to frogs compared to tadpoles. The ability of TDCs to perturb metamorphosis is well documented (Mengeling et al., 2017). Notably, we previously found that exposure of two weeks old *X. laevis* tadpoles to the same TDC mixture used in this study significantly delayed their metamorphosis (McGuire et al., 2021). Here, we further show that TDC exposure prior to metamorphosis can still influence metamorphic processes including immune function, even several months after exposure to the TDCs has stopped. Although little is known about possible persistence and elimination of these TDCs in *Xenopus*, the exposure used was not chronic and performed at relatively low concentration to minimize potential accumulation in tissues. A study of TBBPA metabolism and excretion in *X. laevis* tadpoles found that amphibians excrete into water the same metabolites of TBBPA that are found in mammalian urine samples and upregulate the same xenobiotic metabolizing enzymes in the liver as mammals (Fini et al., 2012). Thus, the long-term effects of transient early life exposures may have significant outcomes on growth and development in *X. laevis*. While exposure to the TDC mixture just before and during metamorphosis rather than at early tadpole stage did not significantly influence the timing of metamorphic completion, it did nevertheless slow the migration of the thymus organ during metamorphosis. In the early stages of post-metamorphic development, the thymus migrates from below the middle part of the ear to the area near the shoulder that is between the scapula and jawbone in juvenile frogs (Jordan & Speidel, 1924). Afterwards in adulthood the thymus progressively involute as aging begins, and thymic output decreases (Plytycz, Mika, & Bigaj, 1995). TDC exposure may still be capable of inducing TH dependent effects on growth and development even when metamorphic timing is altered.

During metamorphosis, a new wave of lymphopoietic progenitor cells colonize the thymus (Turpen & Smith, 1989), (Turpen, 1998). These new thymocyte precursors do not expand until after the metamorphosis completion, although the timing of this expansion and its susceptibility to TDC exposure is unclear. Our data suggest that the lymphopoietic progenitors differentiate and expand in the thymus between 14- and 21-days post-metamorphosis, as there is little detectable thymic output at 14 days post-metamorphosis, whereas there is a strong surge at day 21. Our data also indicate that TDC exposure led to a reduction in immature cortical thymocytes at 21 days post-metamorphosis, while mature CD8 + thymocytes are not significantly decreased. These results correspond to previous published evidence where inhibition of metamorphosis was found to interfere with thymocyte expansion post-metamorphosis, while not impacting hematopoiesis directly (Rollins-Smith & Blair, 1990a). The immature cortical thymocytes notably consist of the bulk of all thymocytes following successive waves of proliferation at the double negative stage. There was no significant difference for the proliferation of these cells. However, most proliferation in thymocytes occurs during the double negative stage, which could not be adequately identified with the available antibodies available in *X. laevis*. It is not known how long mature CD8 + T cells stay in the medulla of the *X. laevis* thymus before entering circulation, so the lack of difference in the mature CD8 + thymocytes may be misleading. Indeed, there was a significant decrease in the number of mature CD8 + T cells that did reach the spleen. Expansion of T cell populations early in life can be highly influential for the establishment of T cell clonal diversity later in life (Gaimann, Nguyen, Desponds, & Mayer, 2020). While there are other T cells found in circulation that are not detectable in the spleen, a lower number of splenic T cells could imply a weaker and more limited T cell repertoire that is less capable of responding to various pathogens.

The defects in CD8 + T cell repopulation in periphery prompted an investigation of whether TDC exposure prior to metamorphosis had lasting impacts on anti-viral response. Notably, anti-viral gene expression in kidney (e.g., TNFα and Type I IFN) was decreased in developmentally exposed frogs, despite no marked changes in FV3 viral loads. Previous studies have shown that in adult frogs an innate immune response characterized by induced expression of genes such as type I IFN and TFNα is detected as early as 1 day following an FV3 infection and peaks at 3 days post-infection, whereas the adaptive CD8 T cell response culminates at 6 days post-infection (Morales & Robert, 2007), (Morales et al., 2010). The weaker induction of type I IFN and TFNα gene expression in developmentally exposed frogs suggests that innate immune cell production of anti-viral cytokines may be impaired, but not sufficiently to enable faster progression of FV3 infection. Previous work using the FV3 infection model has found that there can sometimes be significant changes to the expression of anti-viral cytokines without overt changes in FV3 viral load (Robert et al., 2018). However, it is likely that deficiencies in anti-viral cytokine expression are still biologically impactful because they are important for stimulating the T cell response that is necessary to clear the infection in adult frogs (De Jesús Andino, Chen, Li, Grayfer, & Robert, 2012). Type I IFN triggers intracellular anti-viral defenses to limit FV3 replication in *X. laevis* (Bui-Marinos, Todd, Wasson, Morningstar, & Katzenback, 2021). TNFα is important for the recruitment of innate immune cells to sites of infection and inflammation (Hackel, Aksamit, Bruderek, Lang, & Brandau, 2021). Therefore, small reductions in the production of these cytokines may still weaken resistance to FV3 infection. Furthermore, the lower number of proliferating CD8 T cells in developmentally exposed frogs may lead to a less efficient viral clearance and longer persistence of the viral pathogen. It will be interesting in future experiments to monitor viral loads at later stages of infection (1–2 weeks). Finally, it is possible that the weaker cytokine gene response leads to more extensive tissue damage, which was not assessed in this study (e.g., histology).

Clonal expansion of CD8 + T cells expressing antigen specific TCRs is an important component of the adaptive immune response to viral infections (Fearon, 2007). Our data indicate that developmentally exposed frogs had impaired proliferation of CD8 + T cells. With the tools available for use in *X. laevis*, CD8 + T cells with a TCR specific for FV3 could not be measured. Despite this limitation, the very low amount of proliferative CD8 + T cells, as well as the reduced frequency of CD8 + T cells in the spleen following developmental TDC exposure indicate a dramatic defect of T cell immunity later in life. As the deficiency is still

present at 6 months post-metamorphosis, it is doubtful that the T cell response can fully recover from developmental TDC exposure, especially after thymic involution starts to occur.

Exposure to TDCs prior to metamorphosis reduced the frequency of MHC-II + splenic lymphocytes post-metamorphosis. MHC-II is found on the surface of all adult T cells in *X. laevis*, but is only found on B cells, macrophages, and thymic and splenic epithelial cells in tadpoles (Du Pasquier & Flajnik, 1990). MHC-II expression on T cells is known to be dependent on TH and metamorphosis in *X. laevis* (Rollins-Smith & Blair, 1990b). Therefore, it is likely that the deficiencies in post-metamorphic T cells following TDC exposure are TH-axis dependent, rather than through other pathways that the chemicals may act on. The deficiencies of cell surface MHC-II expression suggest that post-metamorphic lymphocytes may fail to reach maturity, and no longer display the adult T cell phenotype in adult *X. laevis*. Alternatively, it is possible that TDC impairs the expression of the MHC-II gene(s) or protein via the CIITA regulatory gene (León Machado & Steimle, 2021). More mechanistic studies will be needed to elucidate this effect. Nevertheless, the deficiency in surface MHC-II expression detected in this study, coupled with reduced CD8 + T cell renewal after metamorphosis and deficient proliferation during FV3 infection indicate strong impacts of TDCs on the development of T cell immunity in *X. laevis*.

Collectively our data provide evidence of the potential negative impact of TDC exposure during perinatal development on thymocyte differentiation as well as the establishment of an efficient T cell function and antiviral immunity later in life.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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