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Developmental exposure to chemicals associated with unconventional oil and gas extraction alters immune homeostasis and viral immunity of the amphibian *Xenopus*

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ABSTRACT

Although aquatic vertebrates and humans are increasingly exposed to water pollutants associated with unconventional oil and gas extraction (UOG), the long-term effects of these pollutants on immunity remains unclear. We have established the amphibian *Xenopus laevis* and the ranavirus Frog Virus 3 (FV3) as a reliable and sensitive model for evaluating the effects of waterborne pollutants. *X. laevis* tadpoles were exposed to a mixture of equimass amount of UOG chemicals with endocrine disrupting activity (0.1 and 1.0 μg/L) for 3 weeks, and then long-term effects on immune function at steady state and following viral (FV3) infection was assessed after metamorphosis. Notably, developmental exposure to the mixture of UOG chemicals at the tadpole stage affected metamorphic development and fitness by significantly decreasing body mass after metamorphosis completion. Furthermore, developmental exposure to UOGs resulted in perturbation of immune homeostasis in adult frogs, as indicated by significantly decreased number of splenic innate leukocytes, B and T lymphocytes; and a weakened antiviral immune response leading to increased viral load during infection by the ranavirus FV3. These findings suggest that mixture of UOG-associated waterborne endocrine disruptors at low but environmentally–relevant levels have the potential to induce long-lasting alterations of immune function and antiviral immunity in aquatic vertebrates and ultimately human populations.

1. Introduction

Unconventional oil and gas extraction (UOG) has markedly increased production of oil and natural gas in the U.S. over the last 10 years ((Energy Information Administration, 2016; Kassotis et al., 2015a). The process consists of injecting at high pressures millions of gallons of water mixed with sand, and various chemical agents (including acids, friction reducers, and surfactants) into underground shale deposits at high pressures in order to collect trapped oil and natural gas (Carpenter, 2016; Kassotis et al., 2016b; Mrdjen and Lee, 2015). In addition to physical and chemical damage to ecosystems, there is growing concern about negative health impacts on human populations as well as aquatic wild life in regions where UOG is performed. Indeed, among the large number of chemicals associated with UOG (estimated to be over 750) at least 200 have been detected in wastewater, ground water, and surface water (Elsner and Hoelzer, 2016; Vengosh et al., 2014; Waxman et al., 2011; Webb et al., 2014). A number of these chemicals have been shown to be endocrine or developmental disruptors (Casey et al., 2016). Extensive study of water collected at UOG sites has identified certain chemicals that consistently present at concentration ranging from 0.01 to 2.0 mg/L (Gross et al., 2013; Wilkin and Digiulio, 2010). Among these, 23 showed significant agonistic or antagonistic activity *in vitro* for various hormone receptors, including androgen, estrogen, and thyroid, progesterone, and glucocorticoid; and are thus considered endocrine disruptor chemicals (EDCs) (Kassotis et al., 2015b; Kassotis et al., 2014). Because of their wide combined distribution across UOG sites, an equimass mixture of these 23 EDCs has been used to model health risks from exposure to water contaminated by UOG. The rationale is that while each EDC may be present at below an effective concentration, the additive combination of multiple EDCs can induce biological effects, especially when exposure occurs during the more sensitive early developmental period. Indeed, exposure of pregnant mice via

drinking water with this mixture of 23 chemicals at relevant environmental concentrations induces multiple developmental defects in pups including sperm counts and increased testes, body, heart, and thymus weights (Kassotis et al., 2015b). Maternal exposure also induced elevated serum testosterone levels in male pups (Kassotis et al., 2015b) as well as pituitary hormones and mammary gland development in females (Kassotis et al., 2016a; Sapouckey et al., 2018).

Another less well appreciated biological system affected by EDCs is the immune system (Maqbool *et al.* 2016; Vandenberg et al. 2012(Boule and Lawrence, 2016; Kuo et al., 2012). The endocrine system, especially the neuroendocrine axis, is known to play an important role in the development and function of the vertebrate immune system including *Xenopus* (review in (Blom and Ottaviani, 2017; Kinney and Cohen, 2009; Quatrini et al., 2018)). This connection is underlined in metabolic disorders like type 2 diabetes for which EDCs such as bisphenol A and phthalates are considered as promoting factors that affect both endocrine and immune function (Bansal et al., 2018). Notably, early life exposure to several EDCs cause alterations in immune function persisting in adulthood (Boule and Lawrence, 2016). While little is still known about the effects of EDCs associated with UOG, developmental exposure of pregnant mice to similar low doses of the mixture 23 UOG chemicals was found to induce long-term perturbations of the immune system of adult offspring at steady state, and alteration of frequencies of different T cell subsets after immune challenge (Boule et al., 2018). In addition, this developmental exposure to UOG chemicals accentuated immunopathology of experimentally induced of autoimmune encephalitis. Since even modest perturbation of immune function may have decisive consequences on host resistance pathogens, immune alteration potentials of UOG chemical mixture merit further exploration.

We have developed a reliable, sensitive and cost-effective model system based on the amphibian *Xenopus* and the ranavirus FV3, which is an excellent complement to the mouse model to investigate the impact of early life exposure to waterborne mixtures of UOG toxicants on immunity later in life (Gantress et al., 2003; Jacques et al., 2017). *Xenopus* are ideally suited to define the long-term health effects of developmental exposure to waterborne pollutants. The *Xenopus* immune system is extensively characterized and remarkably conserved to that of human *Xenopus* (Robert and Ohta, 2009). Importantly, *Xenopus* are completely aquatic at all stages of development and unlike mammals, develop externally, free of maternal influences. Furthermore, metamorphosis parallels the perinatal period in humans (Fini et al., 2012). Ranavirus pathogens like FV3 (large DNA viruses of the family *Iridoviridae*) have become major viral pathogens, causing infectious diseases and targeting a wide range of aquatic vertebrate species such as amphibians, fish, and reptiles worldwide (Bandin and Dopazo, 2011; Chinchar, 2002; Chinchar et al., 2009; Greer et al., 2005; Jancovich et al., 2010). We have shown that similar to mammals, *Xenopus* adult frogs rely on efficient B and T cell responses activated by innate immune cells to control and clear FV3 infection (Chen and Robert, 2011; De Jesus Andino et al., 2012; Morales et al., 2010). This *Xenopus*/FV3 experimental platform has been useful to reveal that certain herbicides (atrazine) and insecticides (carbaryl) contaminating water at low but ecologically-relevant concentrations induce dramatic acute and long-term persisting defects of anti-FV3 immune responses (De Jesus Andino et al., 2017; Sifkarovski et al., 2014).

To investigate the potential of mixture of EDC water pollutants associated with UOG activity we took advantage of the *Xenopus*/FV3 system. We previously reported that the same mixture of 23 UOG chemicals can affect the immune system of the tadpoles of the amphibian *Xenopus laevis* (Robert et al., 2018). More specifically, a three week exposure of tadpoles to an equimass ranging

from 1 to 0.1 μg/L of the 23 UOG chemicals significantly altered homeostatic expression of myeloid lineage genes. Furthermore, upon infection with the ranavirus FV3, the expression of innate immune response genes TNF-α, IL-1β, and Type I IFN was reduced and the viral loads were increased (Robert et al., 2018). This is of relevance since aquatic animals such as amphibians are continuously exposed to water pollutants and therefore, are likely to become more susceptible to adverse health effects or physiological consequences such as alteration of immune defense mechanisms against ranavirus pathogens.

Here, we have further tested the hypothesis that developmental exposure of tadpoles to current environmental levels of a mixture of 23 UOG chemicals with demonstrable EDC activity result in long lasting developmental defects leading to altered immune homeostasis and antiviral immunity in adult frogs, thus increasing susceptibility to pathogens such as FV3.

2. Materials and Methods

2.1. Animals

All outbred *Xenopus laevis* were from the *X. laevis* research resource for immunology at the University of Rochester (http://www.urmc.rochester.edu/mbi/resources/Xenopus/) following standard husbandry methodology regularly updated by the *Xenopus* community (see: http://www.xenbase.org/entry/). All animals were handled in accordance with stringent laboratory and University Committee on Animal Research regulations (Approval number 100577/2003-151).

2.2. Chemical mixture preparation

144 Twenty-three chemicals (≥97% purity, Sigma Aldrich) were selected based on prior demonstration
145 of endocrine activity, via the estrogen, androgen, progesterone, glucocorticoid, and/or thyroid
146 receptors (Kassotis et al., 2015b; Kassotis et al., 2014). Stock solutions (1 mg/ml) of chemicals
147 were prepared in 100% ethanol (ThermoFisher Scientific, Waltham, MA), stored at -20°C, and
148 used in experiments within 3 months of preparation. The chemicals were: 1,2,4-Trimethylbenzene;
149 2-(2-Methoxyethoxy)ethanol; 2-Ethylhexanol; 2-Methyl-4-isothiazolin-3-one; Acrylamide;
150 Benzene; Bronopol; Cumene; Diethanolamine; Ethoxylated nonylphenol; Ethoxylated
151 octylphenol; Ethylbenzene; Ethylene glycol; Ethylene glycol monobutyl ether; Naphthalen; N,n;
152 Dimethylformamide; Phenol; Propylene glycol; Sodium tetraborate decahydrate (borax); Styrene
153 Toluene; Triethylene glycol; Xylenes.
154
155 *2.3. Animals exposure to water contaminants*
156 Three-weeks old (stage 52, 1.5 cm long; (Nieuwkoop and Faber, 1967)) tadpoles were exposed for
157 3 weeks UOG mixture by diluting an equimass amount of 23 UOG-associated chemicals in the
158 tadpole housing water (dechlorinated water at room temperature [22ºC] and neutral pH 6.8-7.0)
159 from a freshly prepared stock solution at a final concentration of 0.1 and 1.0 µg/mL of each
160 constituent chemical as previously described (Kassotis et al., 2015b; Kassotis et al., 2014). Control
161 tadpoles were kept in water spiked with the vehicle control (0.2% ethanol). Animals were then
162 raised in clean water until 6 months of age. The doses were chosen based on estimates of
163 environmentally relevant oral exposures, such that the two concentrations are similar to levels
164 detected in surface and groundwater in UOG production regions (Cozzarelli et al., 2017; Crosby
165 et al., 2018; DiGiulio and Jackson, 2016; Gross et al., 2013) as well they are lethal to tadpoles
166 (Robert et al., 2018). Animals were raised in a room that is controlled for light cycle (12 hrs.

light/12 hrs. obscurity) and temperature, and has filtered and dechlorinated water. Animals were maintained at a density of 20 tadpoles or 3-5 post-metamorphic froglets per 4 L container. Tadpoles were fed daily with food pellets (Purina Gel Tadpole Diet), adults were fed daily with adult type pellets (Zeigler's Xenopus pellets). While the stability of all 23 chemicals in water is uncertain or unknown, to ensure consistency of exposure, minimize potential degradation and fluctuations in the concentration over time, water and chemicals for each treatment was changed weekly.

2.4. Frog virus 3 stocks and infection

Baby hamster kidney cells (BHK-21, ATCC No. CCL-10) were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), streptomycin (100µg/mL), and penicillin (100 U/mL) with 5% CO2 at 37°C, then 30ºC 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 $1x10^6$ PFU in 10 µL of amphibian PBS (APBS) using a glass Pasteur pipette whose small end had been pulled in a flame (De Jesús Andino et al., 2012). Uninfected control animals were mock-infected with an equivalent volume of APBS. Three and six days post-infection (dpi), animals were euthanized using 0.1% tricaine methanesulfonate (TMS) buffered with bicarbonate prior to dissection and extraction of nucleic acids from tissues (Fig. 1A).

2.5. Quantitative gene expression analyses

Total RNA was extracted from frog kidneys, livers and spleens using Trizol reagent, following the manufacturer's protocol (Invitrogen). cDNA was synthetized with 0.5 µg of RNA in 20 µl using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and 1 µl of cDNA template was used in

all RT-PCRs and 150 ng DNA for PCR. Minus RT controls were included for every reaction. A water-only control was included in each reaction. RNA was checked for purity via nanodrop and only samples that amplified the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) within 20 cycles were used for qPCR analysis. The qPCR analysis was performed using the ABI 7300 real-time PCR system with PerfeCT SYBR Green FastMix, ROX (Quanta) and ABI sequence detection system (SDS) software. GAPDH controls were used in conjunction with the delta^delta CT method to analyze cDNA for gene expression. All primer sequences are listed in Table S1.

2.6 Viral load quantification by qPCR

FV3 viral loads were assessed by absolute qPCR by analysis of isolated DNA in comparison to a serially diluted standard curve. Briefly, an FV3 DNA Pol II PCR fragment was cloned into the pGEM-T Easy vector (Promega). This construct was amplified in bacteria, quantified and serially diluted to yield 10^{10}-10^{1} plasmid copies of the vDNA POL II. These dilutions were employed as a standard curve in subsequent absolute qPCR experiments to derive the viral genome transcript copy numbers, relative to this standard curve.

2.7 Flow cytometry

The *XRRI* provided *X. laevis*-specific monoclonal antibodies (mAbs), including anti-CD5 (1F8), anti-IgM (10A9); anti-NK cell (1F8), anti-class MHCII (AM20) and biotinylated anti-CD8 (AM22) as well as fluorophore-goat-anti-mouse Ab (BD Biosciences) and streptavidin-fluorophore (BioLegend). Splenocytes (2.5×10^5 cells/per treatment) were sequentially stained at 4°C with 100 µl of the different undiluted hybridoma supernatant, secondary goat Abs,

biotinylated anti-CD8 mA and streptavidin according to detailed published protocol (Edholm, 2018). Dead cells were excluded with propidium iodide (BD Pharmingen). 10,000 events per sample, gated on live cells, were collected with Accuri C6 (BD Biosciences). Data was analyzed with FlowJo (TreeStar).

2.8. Statistical analysis

The Mann-Whitney *U* and ANOVA as well as non-parametric Kruskal-Wallis tests were used for statistical analysis of expression and viral load data. Analyses were performed using a Vassar Stat online resource (http://vassarstats.net/utest.html). Statistical analysis of survival data was performed using a Log-Rank Test (GraphPad Prism 6). A probability value of $p<0.05$ was used in all analyses to indicate significance. Error bars on all graphs represent the standard error of the mean (SEM).

3. Results

3.1. Effects of UOG mixture on X. laevis metamorphic development.

Since anuran metamorphosis is under tight endocrine regulation, we first assessed whether developmental exposure to a mixture of UOG chemicals with EDC activity would affect its completion. Pre-metamorphic tadpoles (stage 52) were exposed for 3 weeks to different amount (1 and 0.1 μg/L) of UOG mixture, then transferred to clean water to grow until they metamorphose and reach adult stages (Fig. 1A). These two concentrations match those used in mouse to reflect environmentally relevant exposure (Boule et al., 2018) and are in the low range of the average

concentration for which each of these chemicals has been found in ground water near UOG operations (i.e., 0.01 to 2.0 μg/L; (Gross et al., 2013; Wilkin and Digiulio, 2010)). We did not test higher concentrations (5 and 10 μg/L) because they were shown to induce marked mortality in tadpoles during the exposure (Robert et al., 2018).

Following 3 weeks exposure to 1.0 or 0.1 μg/L of UOG mixture, no significant increase mortality nor gross developmental abnormality (e.g., limb deformation, etc.) was observed over the whole experiment. In particular, there was no increase in mortality during metamorphosis compared to control animals exposed to 0.2% ethanol (Table 1). To refine our analysis, we determined the time in days for each animal treated at tadpole stage to complete metamorphosis, which is defined by the full loss of the tail. There was no statistically significant difference in the time to complete metamorphosis across the different treatment group (Table 1). However, developmental exposure to both 1.0 μg/L and 0.1 μg/L UOG mixture resulted in a statistically significant decrease whole body weight at the end of metamorphosis (Table 1; Fig. 1B). Collectively, these data strongly suggest that UOG chemicals, even at relatively low doses, have the potential to perturb amphibian metamorphic development.

3.2. Effects of developmental exposure to UOG chemicals on splenic leukocytes at steady state

Since *X. laevis* do not have lymph nodes, the spleen is both the primary and main secondary immune organ. As such, we first examined the cellularity and composition of splenocytes in young adult frogs that were exposed to UOG mixture at tadpole stages and were sham-infected by i.p. injection of sterile APBS, which does not elicit detectable innate or adaptive immune responses compared to unmanipulated controls (De Jesus Andino et al., 2017; Morales et al., 2010; Morales and Robert, 2007). Consistent with a decreased whole-body weight, there was a decrease in the

259 total number of cells recovered from the spleen in animals developmentally exposed to UOG
260 chemicals, which was only statistically significant for the group treated with 0.1 μg/L of UOG
261 chemicals (Fig. 2).

262 To determine whether particular leukocyte populations were affected, we conducted a flow
263 cytometry analysis using available *X. laevis*-specific monoclonal antibodies (mAbs) and the gating
264 strategy depicted in Fig. 3. Cell of larger size and granularity corresponding to granulocytes were
265 gated separately (Gate 1, Fig. 3). Although specific markers of innate immune cells of the myeloid
266 lineage are lacking in *Xenopus*, we can obtain useful information be gating on non-lymphocyte
267 events and MHC class II expression. This population was only minimally stained (1-5%) with T
268 and B cell specific mAbs. Lymphocytes, forming a distinct population according to forward and
269 side scatter, were gated out and further separated into total T cells with the anti-pan T cell marker
270 CD5 recognized by 2B1 mAb, then further subdivided using the anti-CD8 mAb AM22 into CD8
271 (CD8+/CD5+) and putative CD4 or CD4-like (CD8neg/CD5+) cells as previously shown (Chida
272 et al., 2011). B cells were detected from the lymphocyte gate with anti-Mu mAb 10A9 as IgM+
273 cells co-expressing the MHC class II marker recognized by the mAb AM20 (Flajnik et al., 1990).
274 NK cells were also examined using the anti-NK cell marker 1F8 (Horton et al., 2000). As expected
275 in adult frogs, all lymphocytes were positively stained with the anti-class II mAb. We used this
276 gating strategy, to calculate the relative frequency and cell number of each of these populations.

277 The splenic granulocyte populations of mock-treated animals segregated into two distinct
278 MHC class II^{low} and II^{high} subsets (Fig. 4A). Interestingly, the MHC class II^{high} subset was
279 significantly ablated in animals developmentally exposed at either 0.1 or 1.0 μg/L dose to the UOG
280 mixture, both in frequency and in cell number (sham-infected panel in Fig. 4B, C). The total
281 number but not the frequency of MHC class II^{low} cells was also diminished in treated groups.

For splenic lymphocytes at steady state, in addition to a slight decreased frequency of B cells, CD5+ T cells and CD+8 T cells (mainly at the 1.0 μg/L dose), the relative numbers of all lymphocyte subsets, were considerably decreased in animals developmentally exposed to both doses of the UOG mixture (see Fig. 5A, 6A).

3.3 Effects of developmental exposure to UOG chemicals on antiviral immune response in the spleen

To further investigate the potential impact of developmental exposure to UOG chemicals on the immune system, we assessed immune response during FV3 infection. Young adults that were exposed to 0.1 and 1.0 μg/L of UOG mixture at tadpole stages, were sham-infected or infected intraperitoneally with $1x10^6$ pfu of FV3. Cellular immune gene and expression response were monitored at 3 and 6 days post-infection, which corresponds to early mainly innate and at the peak of adaptive immune response, respectively.

Since in *Xenopus* the spleen functions both as a primary and secondary lymphoid organ, we first monitored the changes in frequencies and relative numbers of the different cell types by flow cytometry. For the sham-treated 0.2% ethanol exposed control group, antiviral immune response was characterized by an increase frequency of class II^{low} granulocytes at 3 and 6 dpi and a decrease number of class II^{high} cells at 3 dpi (FV3-infected right panel Fig. 4 B, C; statistical significance indicated by #). Interestingly, exposure to 0.1 μg/L and 1.0 μg/L dose of UOG chemicals resulted in a significant deficit in both frequency and numbers of class II^{low} granulocytes at 3 dpi when compared to infected 0.2% ethanol exposed controls (Fig. 4B, C; statistical significance indicated by *). It is noteworthy that this defect occurred before the peak of the adaptive antiviral response in kidneys (Morales and Robert, 2007).

An efficient T cell response, especially CD8 cytotoxic T cells, is critical for viral clearance during a FV3 primary infection (Morales and Robert, 2007; Robert et al., 2005). Therefore, we examined in detail the changes in frequency and cells number of total (CD5+), CD8 and CD4-like T cells in the spleen in infected animals at 3 and 6 dpi (Fig. 5B). Notably, the basal deficit in frequency and numbers of the three different splenic T cell populations resulting from developmental exposure to both doses of the UOG mixture was accentuated during FV3 infection. The defect was already notable at 3 dpi for animals developmentally exposed at the higher 1.0 μg/L doses of UOG mixture. Whether this was due to a lower T cell expansion and/or recruitment in the spleen that functions as the main immune site, remains to be determined. Developmental exposure to UOG chemicals also impaired the kinetics of NK cell response in the spleen at 6 dpi compared to EtOH treated controls, whereas B cell alteration was most notable at 3 dpi and for the 1.0 μg/L group (Fig. 6B).

To further examine the T cell function at steady state (sham-infection) as well as the antiviral T cell response at the site of infection (peritoneal cavity) and at the main site of viral replication (kidney), we determined the relative expression of the key T cell co-receptors CD3ε, CD4 and CD8α (Fig. 7). Interestingly, the basal CD3ε gene expression in the sham-infected group was significantly reduced in the spleen, whereas abnormally high levels of CD4 and CD8β transcripts were detected in kidneys (Fig. 7A). Upon FV3 infection, the expression of the 3 genes markedly increased at 3 dpi in PLs, as well as in kidneys where they remained elevated at 6 dpi. In contrast, there was a drop (10x on average) in the expression of these three T cell genes in the spleen at 3 dpi and to a lesser extent at 6 dpi compared to uninfected controls (Fig. 7B). Notably, developmental exposure to both 0.1 and 1.0 μg/L of the UOG chemical mixture negatively affected the CD8β gene expression in PLs at 3 dpi. This was also reflected by a similar decrease in CD3ε

gene expression, whereas CD4 transcript levels did not show significant alteration. In spleen, gene expression profiling revealed mainly a defect in CD3ε expression. In kidneys, no further alteration of gene expression was detected during FV3 infection (Fig. 7B).

3.4 Effects of developmental exposure to UOG chemicals on antiviral immune response in the kidneys

To obtain further evidence of the long-term impact of developmental exposure to the mixture of UOG chemicals on antiviral immune response, we monitored the expression of a selected set of genes relevant for innate and adaptive immunity in the kidneys. For a number of genes tested involved in innate immune response (e.g., IL-1β or IL-10), the differential expression at steady state and during FV3 infection was not significantly different among treatment groups (Fig. 8A; Table S2). However, the expression response of several genes important for antiviral response was perturbated. Notably, FV3-elicited type I IFN gene expression was significantly reduced at both 3 and 6 dpi in in the kidneys of adult frogs that had been developmentally exposed to the low dose (0.1 μg/mL) of the UOG chemical mixture (Fig. 8C). In contrast, there was an exacerbated gene expression of type II IFN or IFNγ at 3 and 6 dpi and of the pro-inflammatory TNFα at 6 dpi in frogs developmentally exposed to both doses of the UOG mixture (Fig. 8B).

Finally, we assessed whether the alterations of immune response resulting from developmental exposure to the UOG chemical mixture had any consequence in controlling FV3 replication by determining the viral loads. In kidneys, the main site of FV3 replication, viral loads were significantly increased at 6 dpi in frogs developmentally exposed to the high dose (1 μg/L) of the UOG chemical mixture compared to EtOH exposed controls (Fig 8B). The lower viral genome

copy numbers in other tissues including the spleen were not significantly different among the different groups (Table S2).

4. Discussion

In this study we show that the *Xenopus* model system we have developed with FV3 as viral pathogen is reliable and very sensitive for assessing the long-term negative impacts on immune function resulting from exposure during early life to water EDC pollutants associated with UOG activity. Our data provide strong evidence that at concentrations well below or at the level found in water where UOG activity occurs (Energy Information Administration, 2016; Kassotis et al., 2016c), a mixture of UOG chemicals can induce alterations of the immune system that persists for a long time after exposure. Thus, early life exposure leads to change in adulthood that include weakened host resistance to viral pathogens. These results are relevant and raise concern for aquatic vertebrates near UOG sites or downstream from UOG waste water spills. However, owing to the conservation of the immune system across all jawed vertebrates these findings also clearly pertain to human health.

Given that each of these 23 UOG chemicals has been selected because of their EDC activity *in vitro*, it is not really surprising that exposure to a mixture of these chemicals can perturb the overall amphibian development. Indeed, effects on metamorphic development have been reported following exposure to EDCs in X*enopus* (Fini et al., 2012). Given the multiple sources of variability (geography, half-life, concentrations of the various chemicals, time of release, etc.), a defined equimass mixture of these 23 chemicals has been used as a titratable, more controllable

and reliable source of contaminants than raw contaminated water from UOG sites. Using this defined UOG mixture, we found that animals exposed to 1 μg/L and even 0.1 μg/L of the mixture, despite being raised in clean water for months, exhibited persisting developmental alterations characterized by a significant weight loss at the end of metamorphosis. A low weight just after metamorphosis is likely to negatively impact their overall fitness. Even more striking, was the reduction in the number of immune cells of the spleen of adult frogs that were exposed to the UOG mixture at postembryonic stages. Although this decrease was only statistically significant at 0.1 μg/L, the trend was similar at 1 μg/L and affected most cell types of splenic immune cells including myeloid and lymphoid lineage cells. To which extend this lack of strict dose dependent effects of developmental exposure to the UOG mixture is real or just due to high individual variations is unclear. It is to note, however, that non-linear responses are a commonly known attribute of EDCs (Vandenberg et al., 2012). To which extent this immune cell deficit is related to the overall weight loss of animals developmentally exposed to UOG chemicals is unknown. It is also currently unclear whether this decrease in splenic leukocytes/lymphocytes results from a differentiation defect since the spleen is a major lymphoid organ and/or an alteration of immune cell trafficking in the whole organism. Our data are consistent with both possibilities, because we see a decrease in steady state immune cells as well as decreases in responding lymphocytes that are activated in response to the virus. Nevertheless, developmental exposure to the UOG chemical mixtures induced other persisting immune specific alterations at steady state.

Notably, the fraction of innate immune cells expressing high amount of MHC class II molecules at the cell surface was markedly depleted in developmentally treated frogs. In mammals, MHC class II is typically expressed by professional immune cells such as dendritic cells (DCs), monocytes and macrophages. Moreover, the level of MHC class II at the cell surface rapidly

increase upon activation by inflammatory stimuli (reviewed in (Holling et al., 2004; Unanue et al., 2016)). As such, the level of MHC class II can serve as a marker of cell activation. In *X. laevis*, cells of the myeloid lineage including monocytes, macrophages and neutrophils also express surface MHC class II (Du Pasquier and Flajnik, 1990; Edholm, 2018; Rollins-Smith and Blair, 1990). Although little is still little known about DCs in *Xenopus*, a subset of splenic immune cells named XL cells has recently been characterized that exhibit dual DC and follicular Dendritic (FDCs) characteristics, and that expresses high level of MHC class II (Neely et al., 2018). Furthermore, indirect evidence suggests that as in mammals, there is increased MHC class II surface expression on activated splenic leukocytes (macrophage and putative DCs) during an immune response (Morales et al., 2010). Thus, the marked decline in the innate cell population expressing a high level of MHC class II may indicate some impairment in leukocyte activation. The persistence of lower frequency and number of MHC class II^{high} leukocytes in the spleen following viral infection further supports this possibility. A less effective activation of these innate cell effectors may have negative impact not only on antiviral innate immune response (e.g., production of inflammatory cytokines) but also on the adaptive immune response by reducing antigen presentation and co-stimulation, which ultimately would delay or decrease B and T lymphocyte activation.

Consistent with this, our data indicate that developmental exposure to UOG chemicals resulted in multiple T cell functional deficits. The lower frequency and number of T cells at 6 dpi in the spleen strongly suggests a defect in T cell expansion, which would be consistent with a poor T cell activation by APCs. Lymphocyte expansion in the spleen during FV3 infection is also well documented (Morales and Robert, 2007). While the role of CD4 T cells in antiviral immunity is currently unknown, CD8 T cells that are crucial for viral clearance during a primary infection with

FV3 (Morales and Robert, 2007; Robert et al., 2005). Besides an impaired activation in the spleen, our data also suggest an alteration in T cell recruitment at the site of infection. This is based on the relative levels of CD3, CD4 and CD8β receptor transcripts used as proxy to detect total, CD4 and CD8 T cells. The change of expression of these genes in the spleen is overall consistent with the flow cytometry indicating a poor T cell activation and/or expansion, which provide some validity for the approach. Notably, our qPCR data suggest that there was delay in infiltration of CD8 T cells at 3 dpi in UOG-treated compared to sham-exposed controls. In kidneys, where viral replication is the most prominent, although little difference was observed with regards to T cell occurrence, altered expression of several cytokine encoding genes was observed. Interestingly, IFNγ gene expression response to FV3 infection appeared to be exacerbated in animals developmentally exposed to both doses of UOG chemicals. This again suggests some deregulation of the T cell response. Owing to the demonstrated importance of CD8 T cell as well as innate-like T cells in anti-FV3 response, it will be useful in future experiments to determine the expression of classical MHC class I and MHC class I-like (Edholm et al., 2013).

In addition to T cells, developmental exposure to UOG chemicals had also lasting negative effects on NK and B cell responses in the spleen. Owing to the lack of information about their development and function, it is unclear whether the lower frequency and number of NK cells during FV3 infection in animal developmentally exposed to 1 μg/L of UOG chemicals is due to an impairment of their differentiation or their recruitment in the spleen. Concerning B cells, their significant lower frequency and number at 3 dpi suggests a reduced B cell response to FV3 in UOG treated animals, which could be related to an ineffective activation as discussed for T cells. It will be interesting in future experiments to assess the isotypes, magnitude and affinity of

antibodies produced during primary and secondary FV3 infection. While there is a B cell response during primary FV3 infections, the thymus-dependent switch from IgM to IgY antibody and the production of high titer of neutralizing IgY in the serum occurs mainly during a secondary FV3 infection (Maniero et al., 2006).

Aquatic vertebrates and human populations are exposed to an increasing number of EDC water pollutants, whose potential long-term harmful effects on immune function are unclear and understudied. The relative sensitivity of *Xenopus* to waterborne contaminants compared to mice and human is a complex issue since for some chemicals (e.g. TCDD; (Lavine et al., 2005)), *Xenopus* is less sensitive than mouse, whereas *Xenopus* is more sensitive than mammals and fish for other chemicals (e.g., phenols; (Lavine et al., 2005)), In addition, amphibians can adapt and become more resistant to a certain pollutants (Hua et al., 2015; Lavine et al., 2005). Nevertheless, while more work will be needed to define the molecular and cellular pathways targeted by mixtures of EDCs derived from UOG activity, our results provide unequivocal evidence of the long term negative impacts on immune function and immune defenses to pathogens that short perinatal exposure to these water pollutants can induce.

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FIGURE LEGENDS

Figure 1. (A) Schematic of developmental treatment strategy. After 3 weeks exposure to the UOG chemical mixture, tadpoles were raised in clean water until 6 month of age, past metamorphosis. (B) Individual and averages ±SD of whole body weights in mg at metamorphosis completion (developmental stage 66) for each treatment group. * $P<0.05$ significant differences relative to EtOH treated only controls using one-way ANOVA test and Tukey's post-hoc test (GraphPad Prism 6).

Figure 2. Total number of leukocytes recovered from the spleen and peritoneal cavity from adult frogs that were exposed for three weeks at tadpole stages to 0.2 % ethanol (Ctrl; black) or 0.1 (gray) or 1 μg/L (white) of an equimass mixture of 23 UOG chemicals. After chemical exposure and metamorphosis completion, frogs were either i.p. injected with 1 $x10^6$ pfu of FV3 or sham-infected with amphibian PBS (steady state), and then euthanized after 1, 3, or 6 days or sham-infected (C; steady state). Results are means ± SEM of 6 individuals per group from two different experiments (3 per experiment). * $P<0.05$ significant differences among treatment groups relative to the EtOH treated group; # $P<0.05$ determined between each corresponding mock-infected and FV3-infected treatment group. All the values were determined by one-way ANOVA test and Tukey's post-hoc test (GraphPad Prism 6).

Figure 3. Flow cytometry gating strategy (Flowjo 7). (A) Representative FACS bit map from splenocytes of a single animal: Y-axis (SSC-A): side light scatter (cell granularity and density). X-axis (FSC-A): light scatter (cell size). Two major cell populations were gated: (1) Larger cells (Mθ, DCs, PMN). (2) Smaller lymphocytes (B and T cells). Splenocytes were stained with mAbs

667 specific for (C) MHC class II; (E) IgM/class II; (F) CD5/CD8 to identify CD8 and CD4 T cells
668 (CD5+/CD8neg; (G) NK cells mAb. A negative control (B) was included for each staining.
669
670 **Figure 4.** Effects of developmental exposure to an equimass mixture of 23 UOG chemicals on
671 myeloid lineage cells at steady state (sham-infected) and during viral infection. (A) MHC class II
672 surface expression by FACS on splenic granulocytes (gate 1) of frogs exposed to 0.2 % EtOH
673 (Ctrl; black), 0.1 (gray) or 1.0 μg/L (white) of UOGs. (B) Frequencies (%) and (C) cell numbers
674 of class II^{high} and class II^{low} granulocytes from UOG exposed frogs either infected with 1 $x10^6$
675 pfu of FV3 for 3 or 6 days, or sham-infected. These data are pools of 2 independent experiments
676 (3 animals per group). * P <0.05 significant differences among treatment groups relative to the
677 EtOH treated group; # P <0.05 significant differences determined between each corresponding
678 mock-infected and FV3-infected treatment group. All the values were determined by one-way
679 ANOVA test and Tukey's post-hoc test (GraphPad Prism 6).
680
681 **Figure 5.** Effects of developmental exposure to an equimass mixture of 23 UOG chemicals on T
682 cells at steady state (sham-infected) (A) and during viral infection (B). Frequency (%) and relative
683 number of splenic total (CD5+), CD8 (CD5+/CD8+/CD5) and CD4-like (CD5+/CD8neg)
684 determined by flow cytometry (gate 2) in frogs exposed to 0.2 % ethanol (Ctrl; black) or 0.1(gray),
685 and 1.0 μg/L (white) of UOGs. *Xenopus*-specific mAb used were: AM20 (class II); 2B1 (CD5);
686 AM22 (CD8). After chemical exposure and metamorphosis, frogs were either i.p. injected with 1
687 $x10^6$ pfu of FV3 or with amphibian PBS (Ctrl), and then euthanized after 3 and 6 d. Results are
688 means ± SEM of 6 individuals per group from two different experiments (3 per experiment). * P
689 <0.05 significant differences among treatment groups relative to the ethanol treated group; # P

690 <0.05 significant differences for each infected group relative to uninfected controls. All the values
691 were determined by one-way ANOVA test and Tukey's post-hoc test (GraphPad Prism 6).
692
693 **Figure 6.** Effects of developmental exposure to an equimass mixture of 23 UOG chemicals on B
694 and NK cells at steady state (sham-infected) (A) and during viral infection (B). Frequency and
695 relative number of splenic IgM+ B cells and NK cells determined by flow cytometry (gate 2) in
696 frogs developmentally exposed to 0.2 % ethanol (Ctrl; black) or 0.1 (gray) and 1.0 μg/L (white) of
697 UOGs then either infected with FV3 for 3 or 6 days, or sham-infected. *Xenopus*-specific mAb used
698 are: AM20 (class II); 10A9 (IgM); 1F8 (NK). These data are pools of 2 independent experiments
699 (3 animals per group). * P <0.05 significant differences among treatment groups relative to the
700 EtOH treated group; # P <0.05 significant differences for each infected group relative to uninfected
701 controls. All the values were determined by one-way ANOVA test and Tukey's post-hoc test
702 (GraphPad Prism 6).
703
704 **Figure 7.** Effects of developmental exposure to UOG chemicals on relative expression of T cell
705 co-receptor CD3, CD4 and CD8 genes at steady state (sham-infected) (A) and during viral
706 infection (B). Relative expression of CD3, CD4 and CD8 genes from the peritoneum, spleen and
707 kidney tissues was determined for adult frogs developmentally exposed to either 0.2% ethanol
708 (Ctrl; black), 0.1 (gray) or 1.0 μg/L (white) of the UOG mixture. After chemical exposure and
709 metamorphosis completion, frogs were either sham-infected or i.p. infected with 1 $x10^6$ pfu of
710 FV3, for 3 and 6 days. Results are means ± SEM of 6 individuals per group from two different
711 experiments (3 per experiment). Gene expression is represented as fold increase (RQ: relative
712 quantification) relative to GAPDH endogenous control. Statistical significance was assessed by

713 Kruskal-Wallis non-parametric test and Dunn's multiple comparison test: (*) P<0.05 between
714 control and treated groups.
715
716 **Figure 8.** Effects of developmental exposure to UOG chemicals on viral load (A) as well as
717 relative expression of TNFα, Type I and II IFN genes in kidneys at steady state (sham-infected)
718 (B) and during viral infection (C). (A) FV3 genome copy numbers in kidneys of infected adult
719 frogs at 6 dpi that were developmentally exposed to either 0.2% ethanol (Ctrl; black), 0.1 (gray)
720 or 1.0 µg/L (white) UOG mixture. For each group, the viral genome copy number of each
721 individual determined by absolute qPCR is depicted by different symbol as well as a horizontal
722 barre indicating the average ±SD. Statistical significance: ** P<0.005 (Kruskal-Wallis non-
723 parametric test and Dunn's multiple comparison test). (B, C) The relative expression of TNFα,
724 Type I and II IFN genes in kidney was determined for adult frogs developmentally exposed to
725 either 0.2% ethanol (Ctrl), 0.1 or 1.0 µg/L of the UOG mixture and either sham-infected or i.p.
726 infected with 1 x10^6 pfu of FV3 for 3 and 6 days. Results are means ± SEM of 6 individuals per
727 group from two different experiments (3 per experiment). Gene expression is represented as fold
728 increase (RQ: relative quantification) relative to GAPDH endogenous control. Statistical
729 significance assessed by Kruskal-Wallis non-parametric test and Dunn's multiple comparison test:
730 (*) P<0.05 between control and treated groups.
731
732

Highlights

- Effects of unconventional oil and gas mixture of chemicals (UOG) in *Xenopus* are presented
- Developmental exposure to 23 UG chemicals (UOG-mix) affects adult frog immunity in adult frogs
- UOG-mix alters immune adult frog homeostasis
- UOG-mix applied to tadpoles weakens frog antiviral immunity after metamorphosis

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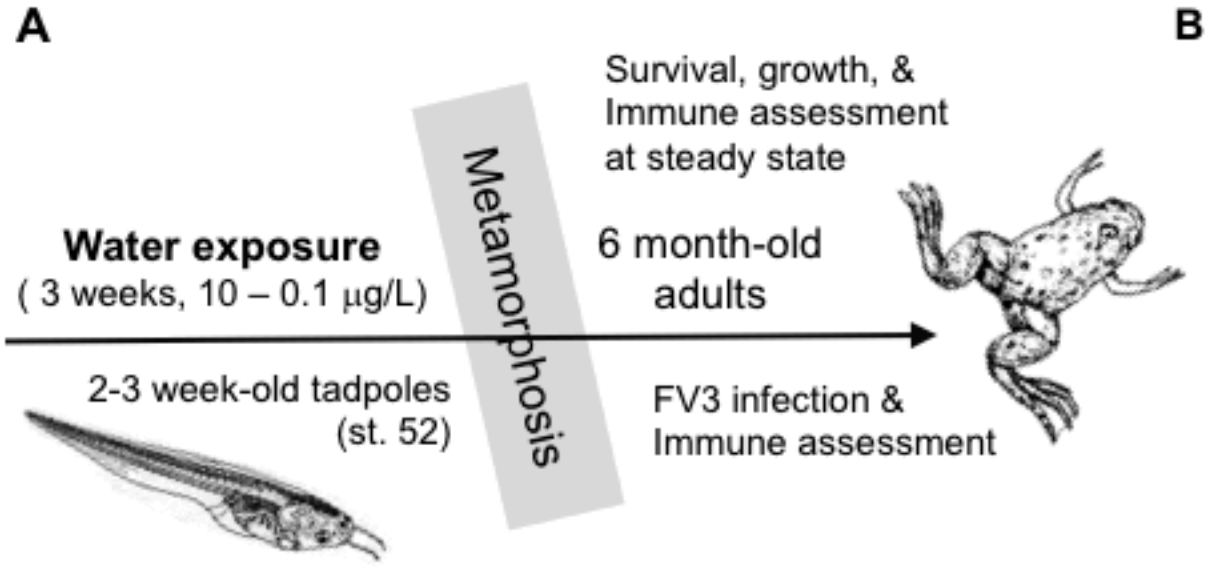
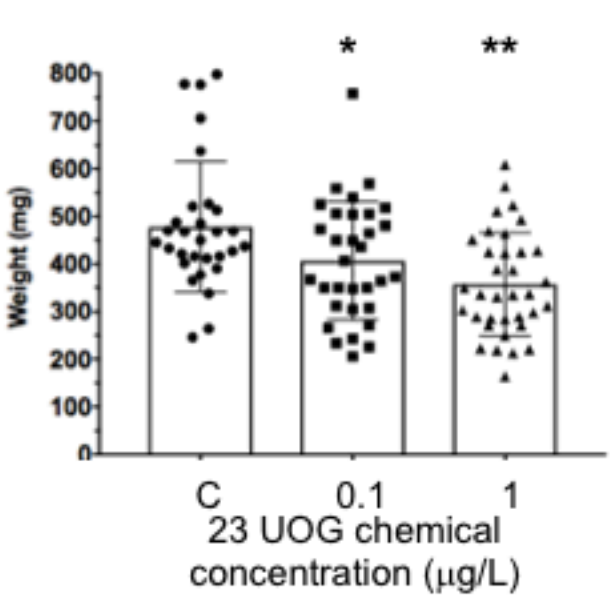
Fig. 1


A
Survival, growth, &
Immune assessment
at steady state
Water exposure
(3 weeks, 10 – 0.1 μg/L)
Metamorphosis
6 month-old
adults
2-3 week-old tadpoles
(st. 52)
FV3 infection &
Immune assessment


B
*
**
Weight (mg)
800
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1
23 UOG chemical
concentration (μg/L)

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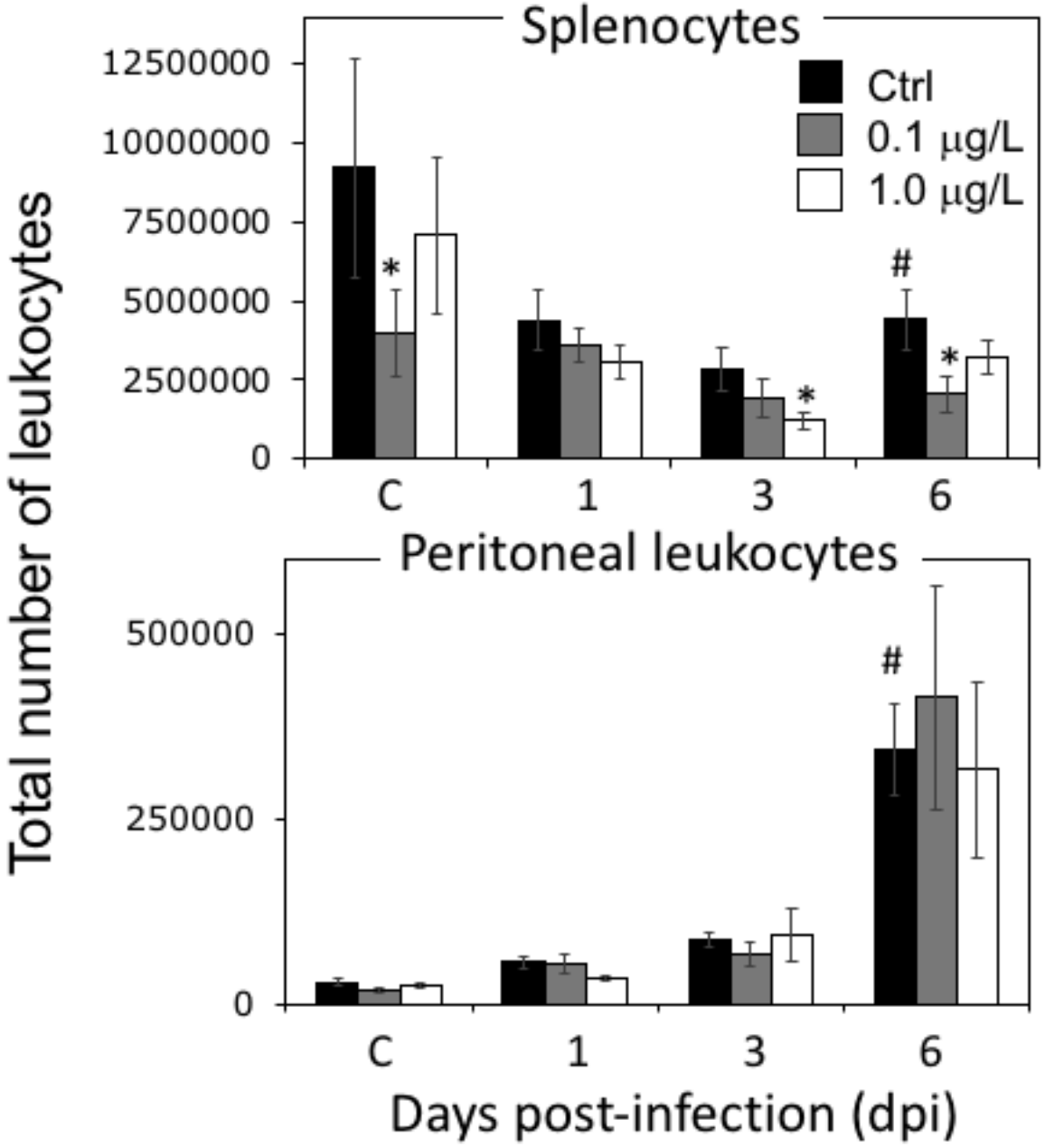
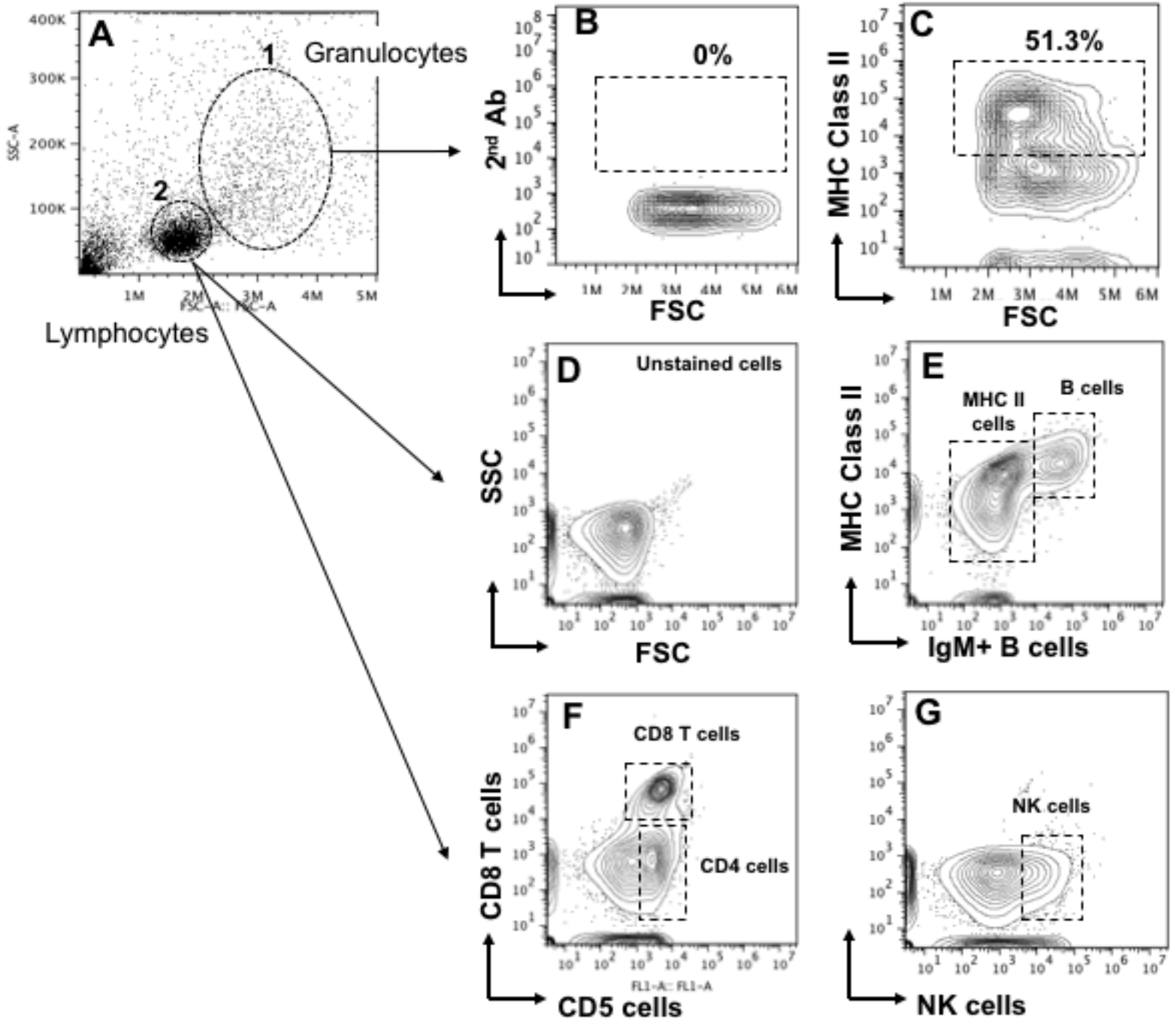
Filename: Slide02.tiff

Fig. 2


Splenocytes
Ctrl
0.1 μg/L
1.0 μg/L
12500000
10000000
7500000
5000000
2500000
0
*
*
*
#
C
1
3
6
Peritoneal leukocytes
500000
250000
0
#
C
1
3
6
Total number of leukocytes
Days post-infection (dpi)

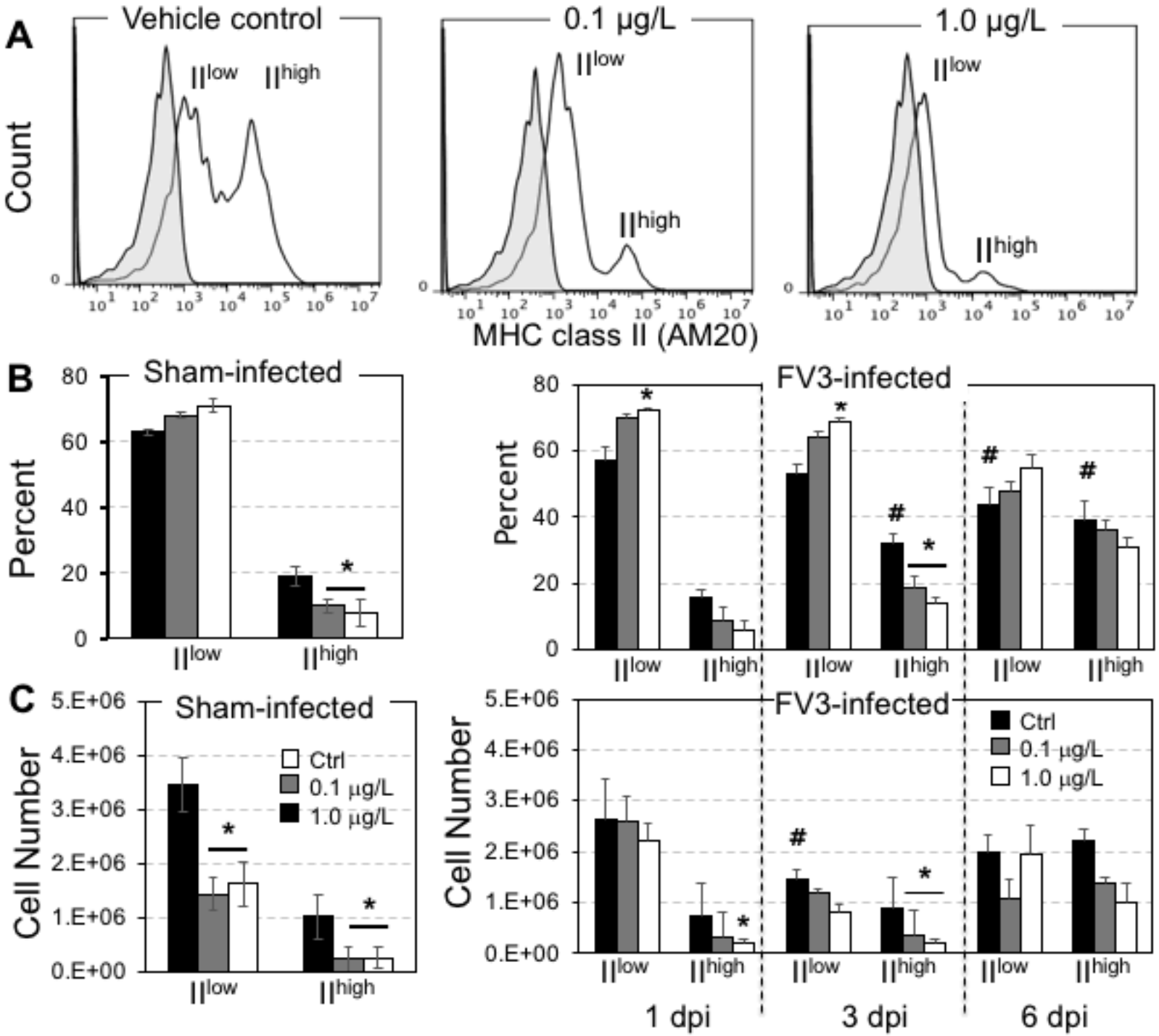
Type of file: figure
Label: 3
Filename: Slide03.tiff

Fig. 3


A
1
Granulocytes
2
Lymphocytes
SSC-A
FSC-A
B
0%
2nd Ab
FSC
C
51.3%
MHC Class II
FSC
D
Unstained cells
SSC
FSC
E
MHC II cells
B cells
MHC Class II
IgM+ B cells
F
CD8 T cells
CD4 cells
CD8 T cells
CD5 cells
G
NK cells
NK cells

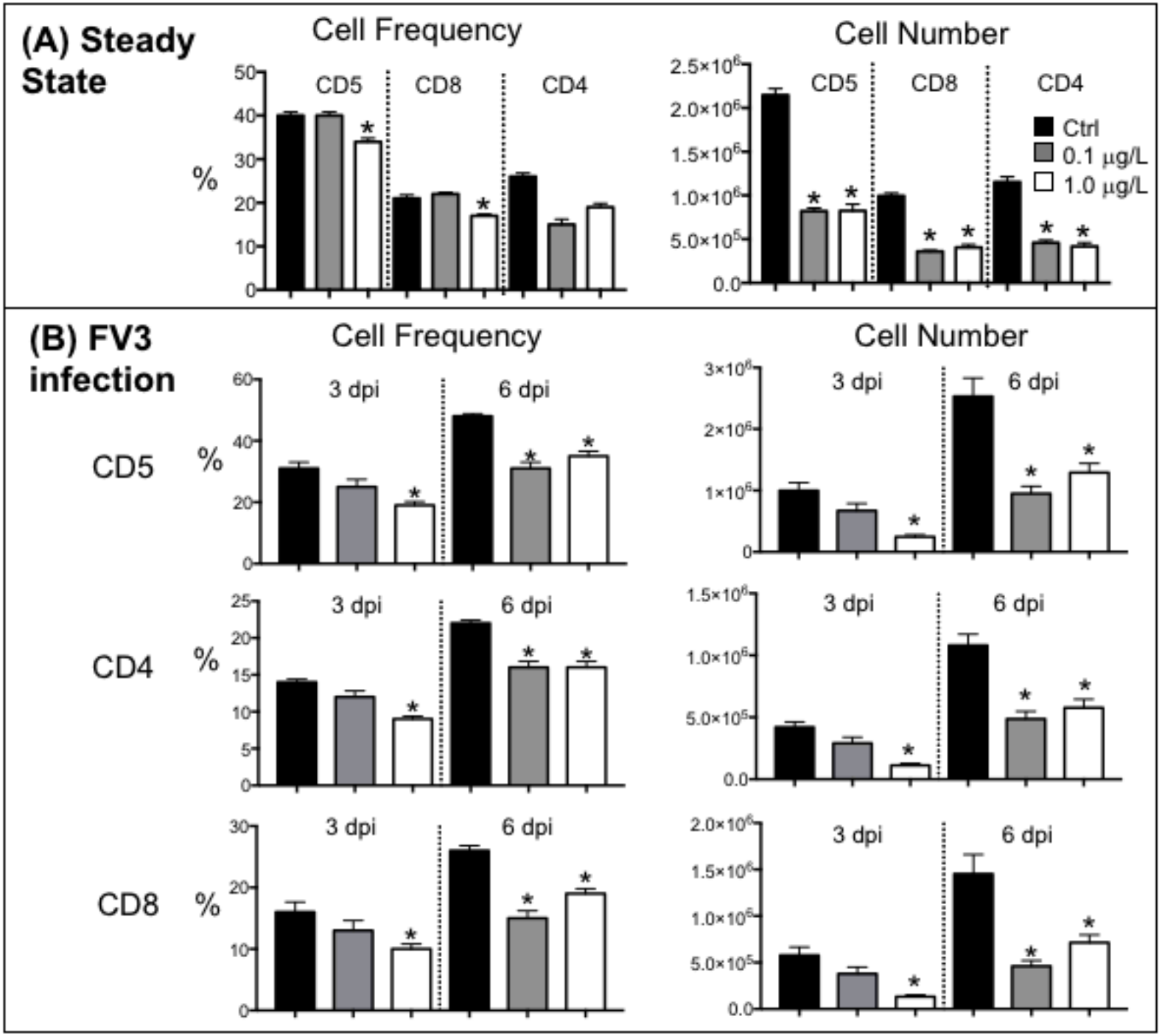
Type of file: figure
Label: 4
Filename: Slide04.tiff

Fig. 4


A
Vehicle control
0.1 µg/L
1.0 µg/L
Count
IIlow
IIhigh
MHC class II (AM20)
B
Sham-infected
FV3-infected
Percent
C
Sham-infected
FV3-infected
Cell Number
Ctrl
0.1 µg/L
1.0 µg/L
1 dpi
3 dpi
6 dpi

Type of file: figure
Label: 5
Filename: Slide05.tiff

Fig. 5


(A) Steady State
Cell Frequency
Cell Number
CD5
CD8
CD4
%
Ctrl
0.1 μg/L
1.0 μg/L
(B) FV3 infection
3 dpi
6 dpi
CD5
CD4
CD8

Type of file: figure
Label: 6
Filename: Slide06.tiff

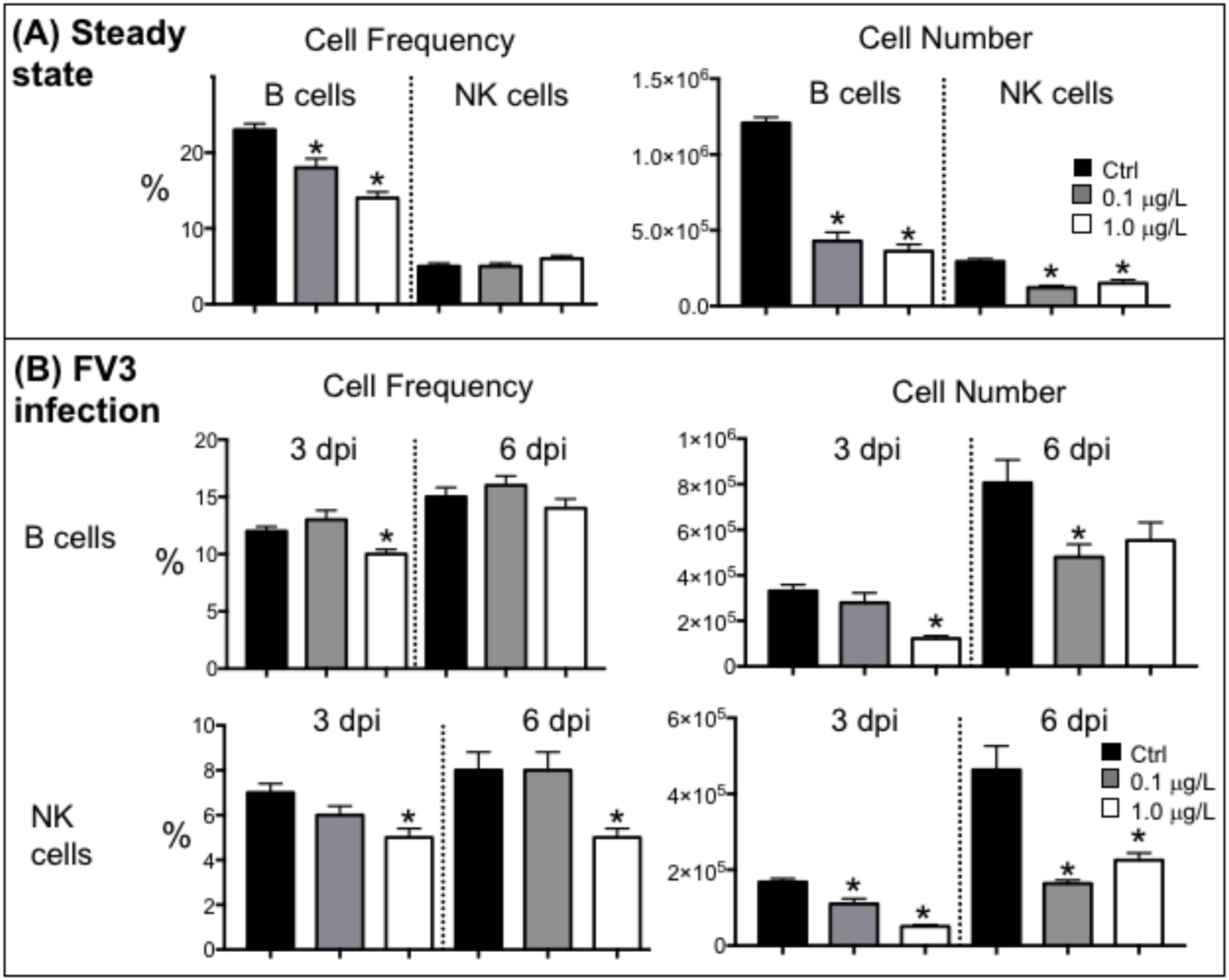

(A) Steady state
Cell Frequency
B cells
NK cells
%
20
10
0
Cell Number
1.5×10^6
1.0×10^6
5.0×10^5
0.0
Ctrl
0.1 μg/L
1.0 μg/L
(B) FV3 infection
Cell Frequency
Cell Number
B cells
NK cells
3 dpi
6 dpi
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
10
8
6
4
2
Ctrl
0.1 μg/L
1.0 μg/L

Fig 6

Type of file: figure
Label: 7
Filename: Slide07.tiff

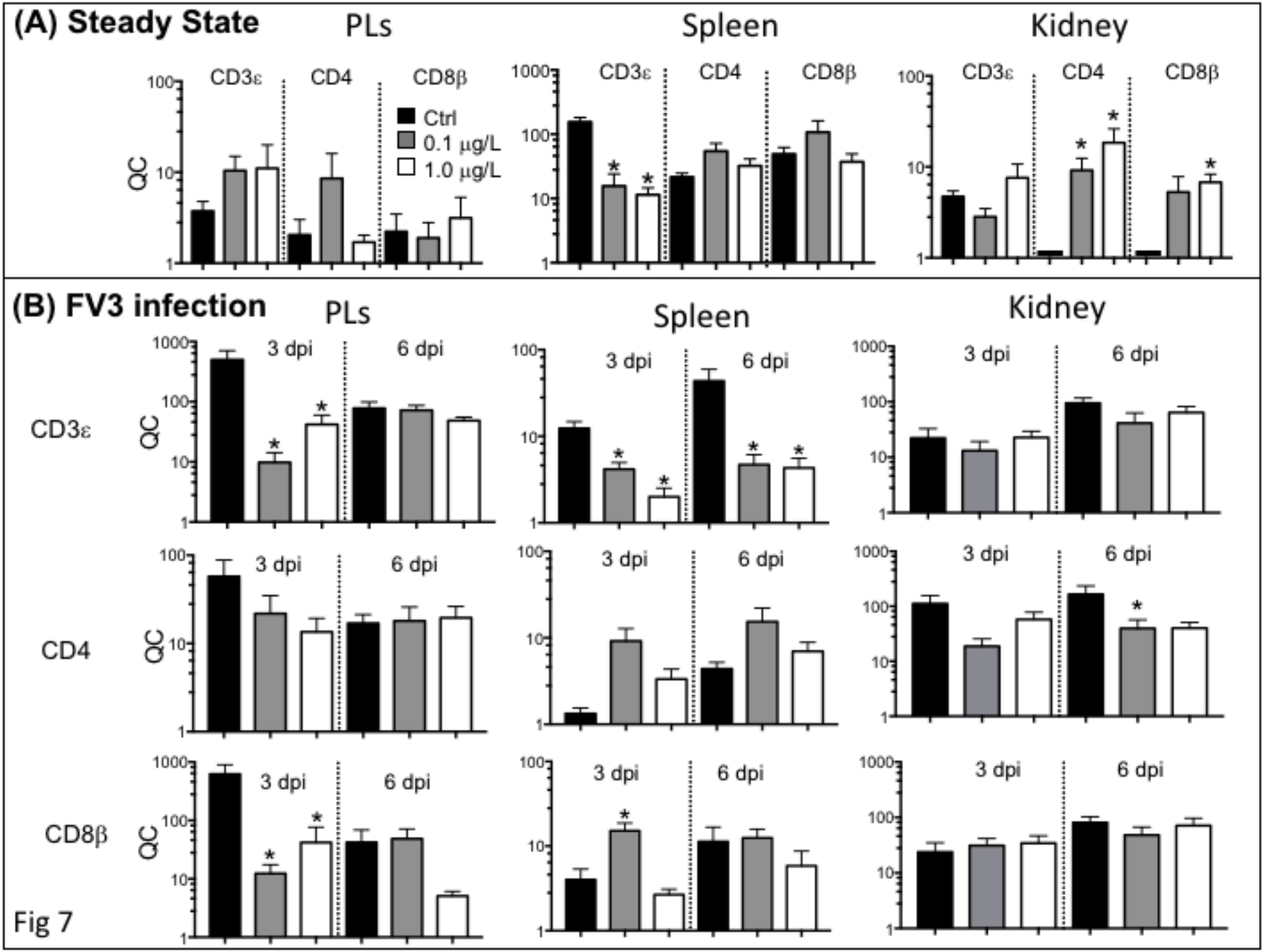
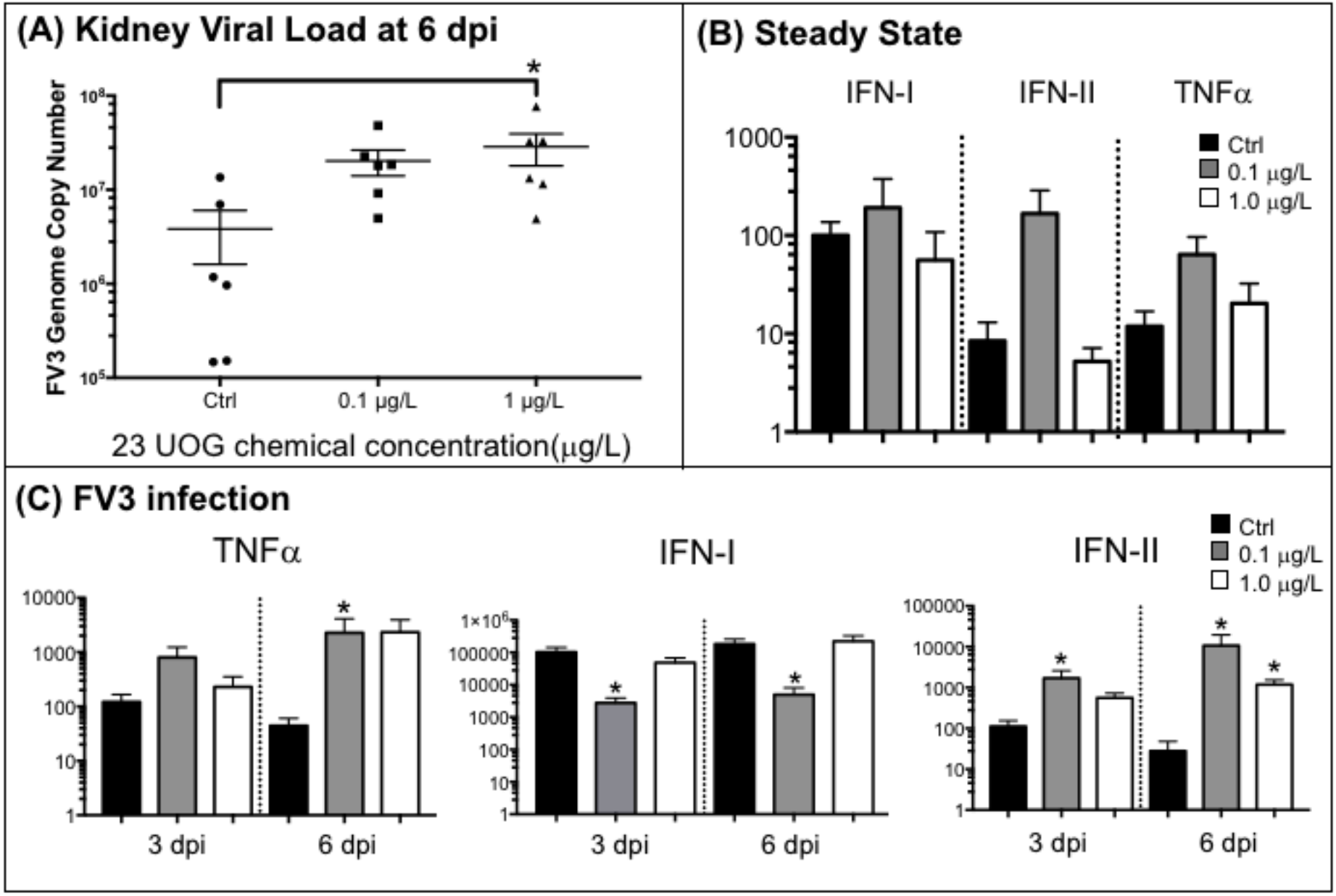

(A) Steady State
PLs
Spleen
Kidney
CD3ε
CD4
CD8β
Ctrl
0.1 μg/L
1.0 μg/L
QC
(B) FV3 infection
3 dpi
6 dpi
CD3ε
CD4
CD8β
QC

Fig 7

Type of file: figure
Label: 8
Filename: Slide08.tiff

Fig 8


(A) Kidney Viral Load at 6 dpi
FV3 Genome Copy Number
Ctrl
0.1 μg/L
1 μg/L
23 UOG chemical concentration(μg/L)
(B) Steady State
IFN-I
IFN-II
TNFα
Ctrl
0.1 μg/L
1.0 μg/L
(C) FV3 infection
TNFα
IFN-I
IFN-II
3 dpi
6 dpi

Type of file: table
Label: 1
Filename: Table 1.doc

Table 1: Survival, body weight and median time to reach metamorphosis completion (stage 66) following UOG treatment.

	Treatment		
Measurements	**Vehicle control**	**0.1 µg/L**	**1 µg/L**
Number of animals	30	33	35
Number of dead animals after treatment	4 (13%)	4 (12%)	5 (14%)
Median time (wks.) to reach stage 66	25	19	16
Weight (mg)	478±25	401±22 p>0.04	357±18 p>0.0002

P value determined between vehicle control and UOG-treated animals using one-way ANOVA test and Tukey's post-hoc test (GraphPad Prism 6).

The NIHMS has received the file 'Supplementary material.doc' as supplementary data. The file will not appear in this PDF Receipt, but it will be linked to the web version of your manuscript.