

Developmental Exposure to a Mixture of 23 Chemicals Associated With Unconventional Oil and Gas Operations Alters the Immune System of Mice

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ABSTRACT

Chemicals used in unconventional oil and gas (UOG) operations have the potential to cause adverse biological effects, but this has not been thoroughly evaluated. A notable knowledge gap is their impact on development and function of the immune system. Herein, we report an investigation of whether developmental exposure to a mixture of chemicals associated with UOG operations affects the development and function of the immune system. We used a previously characterized mixture of 23 chemicals associated with UOG, and which was demonstrated to affect reproductive and developmental endpoints in mice. C57Bl/6 mice were maintained throughout pregnancy and during lactation on water containing two concentrations of this 23-chemical mixture, and the immune system of male and female adult offspring was assessed. We comprehensively examined the cellularity of primary and secondary immune organs, and used three different disease models to probe potential immune effects: house dust mite-induced allergic airway disease, influenza A virus infection, and experimental autoimmune encephalomyelitis (EAE). In all three disease models, developmental exposure altered frequencies of certain T cell sub-populations in female, but not male, offspring. Additionally, in the EAE model disease onset occurred earlier and was more severe in females. Our findings indicate that developmental exposure to this mixture had persistent immunological effects that differed by sex, and exacerbated responses in an experimental model of autoimmune encephalitis. These observations suggest that developmental exposure to complex mixtures of water contaminants, such as those derived from UOG operations, could contribute to immune dysregulation and disease later in life.

Key words: water pollutants; immunotoxicity; hydrofracking; influenza; autoimmune; allergy.

Unconventional oil and gas (UOG) extraction combines hydraulic fracturing with horizontal drilling, and has unlocked oil and gas reserves that, until recently, were inaccessible. In this

process, millions of gallons of water containing proprietary mixtures of chemicals are injected underground under very high pressure. This fractures the shale or coal bed layer, releasing

trapped natural gas and oil (Vengosh *et al.*, 2014; Wiseman, 2009). Over 1000 chemicals have been reported by the industry to be utilized in UOG operations, and over 200 of these have been independently measured in UOG wastewater, as well as surface and groundwater in UOG drilling-dense regions (Elsner and Hoelzer, 2016; United States Environmental Protection Agency, 2015; Vengosh *et al.*, 2014; Waxman *et al.*, 2011; Webb *et al.*, 2014). Little is known about the potential health effects of exposure to water that is inadvertently contaminated with chemicals used in UOG. The scant nature of information hampers the ability to make informed decisions in order to reduce potential effects on human health and prevent unintentional deleterious impacts on complex ecosystems that sustain local economies and our natural environment.

Although research is limited, several epidemiological studies have reported adverse health metrics associated with proximity to UOG activity. A recent systematic review critically evaluated the levels of confidence and evidence for impacts of UOG operations on human reproduction, and found moderate evidence for an increased risk of preterm birth, miscarriage, birth defects, decreased semen quality, and prostate cancer (Balise *et al.*, 2016). Other studies of health outcomes associated with exposures defined by proximity to UOG activities have reported both positive and null associations for preterm birth, low birth weight, and small for gestational age births (Casey *et al.*, 2016; Stacy *et al.*, 2015). An evaluation of 124 842 birth records in Colorado revealed an association between maternal residential proximity to gas development operations and congenital heart and neural tube birth defects among infants (McKenzie *et al.*, 2014). In addition to developmental health outcomes, a positive correlation between residential proximity to oil and gas wells and acute lymphocytic leukemia, but not non-Hodgkin lymphoma, was reported in a case-control study of children and young adults (McKenzie *et al.*, 2017). Associations between UOG operations and asthma exacerbations (Rasmussen *et al.*, 2016), and increases in self-reported upper respiratory symptoms have also been reported (McKenzie *et al.*, 2014; Rabinowitz *et al.*, 2015). Collectively, these reports suggest human health impacts, although there remains uncertainty about potential adverse health effects of UOG operations. Factors that contribute to this uncertainty include: (1) limited information about which diseases to study in humans (or animals) living in regions with UOG activity; (2) the need to identify specific water contaminants and estimate exposures that might result from UOG activities; and (3) lack of research on the effects of chemicals associated with UOG operations using validated experimental systems that model common human diseases.

One category of compounds that have been identified in water near sites with active UOG operations are endocrine disrupting chemicals (EDCs). EDCs are broadly defined as exogenous compounds that singly, or as mixtures, mimic or interfere with the normal actions of hormones (Kassotis *et al.*, 2016b; Maqbool *et al.*, 2016; Vandenberg *et al.*, 2012; Zoeller *et al.*, 2012). A combination of *in vitro* and *in vivo* approaches recently revealed endocrine activity of 23 chemicals used in UOG extraction, and demonstrated antagonism of the estrogen, androgen, progesterone, glucocorticoid, and thyroid receptors *in vitro* (Kassotis *et al.*, 2014, 2015). Maternal exposure of mice to an equimass mixture of these 23 chemicals negatively affected development of male and female reproductive organs, and reproductive parameters such as hormone concentrations, sperm quality, and ovarian follicle development in C57Bl/6 offspring (Kassotis *et al.*, 2014, 2015, 2016a). EDCs can also affect other physiological systems, including the immune system (Boule and Lawrence,

2016; Kassotis *et al.*, 2016b; Kuo *et al.*, 2012; Maqbool *et al.*, 2016; Vandenberg *et al.*, 2012), and early life exposure to several EDCs cause persistent alterations in immune function (Boule and Lawrence, 2016). Yet, little is known about the effects of developmental exposure to chemicals associated with UOG on the development or function of the mammalian immune system.

In rodents as in humans, immune system ontogeny begins in the womb, but continues after birth (Ciau-Uitz *et al.*, 2014). The immune system is critical for maintaining host defense against pathogens, whereas simultaneously self-regulating to avoid immune-mediated tissue damage, autoimmune diseases, and allergic reactions. This is orchestrated by complex and tightly regulated interactions involving many types of immune cells, all of which arise from hematopoietic stem cells (HSCs) and lineage-committed progenitors. Imbalances in immune function can result in diminished ability to fight infections, or can manifest increased hypersensitivities and autoimmune diseases. To establish whether developmental exposure to chemicals associated with UOG could affect the immune system of adult offspring, we studied the same chemical mixture that was previously shown to alter the reproductive organs of male and female mice (Kassotis *et al.*, 2015, 2016a). We characterized the impact of early life exposure to this mixture on the development of primary and secondary immune organs, and compared the effects between male and female offspring. To determine whether this exposure affects functional properties of the adaptive immune system, we focused on T cell responses. CD8⁺ T cells are essential for clearing intracellular infections, including many caused by viruses (Tscharke *et al.*, 2015). CD4⁺ T cells are important for defenses against extracellular pathogens, and drive pathogenesis of immune-mediated diseases, such as allergic airway inflammation and autoimmune diseases (Sun and Zhang, 2014). Depending on the signals they receive during activation, CD4⁺ T cells differentiate into conventional subsets, including Th1 cells, Th2 cells, and Th17 cells, (Hirahara and Nakayama, 2016; Yamane and Paul, 2013). Another sub-type of CD4⁺ T cells, called regulatory T cells or Tregs, play a critical role in dampening immune responses (Josefowicz *et al.*, 2012). Changes to T cell populations provide a metric of adaptive immune function. Therefore, we determined the consequences of developmental exposure to a mixture of chemicals associated with UOG on male and female offspring using three established models of distinct human diseases that require T cells: allergic airway disease (induced using house dust mite [HDM] extract), infectious disease (influenza A virus, IAV), and autoimmune disease (experimental autoimmune encephalomyelitis, EAE).

MATERIALS AND METHODS

Chemical mixture preparation. Twenty-three chemicals ($\geq 97\%$ purity, Sigma Aldrich) were selected based on developmental effects on other physiological systems, and prior demonstration of endocrine activity via estrogen, progesterone, glucocorticoid, and/or thyroid receptors (Kassotis *et al.*, 2015, 2016a). They are also among chemicals used in UOG operations and detected in surface and groundwater in UOG drilling-dense regions (Colborn *et al.*, 2011; United States Environmental Protection Agency, 2015; Waxman *et al.*, 2011). Stock solutions of chemicals were prepared in 100% ethanol (ThermoFisher Scientific, Waltham, MA), stored at -20°C , and used in experiments within 6 months of preparation.

Mice and developmental exposure. Adult (6-week old) male and female C57Bl/6 mice were purchased from Jackson Laboratories

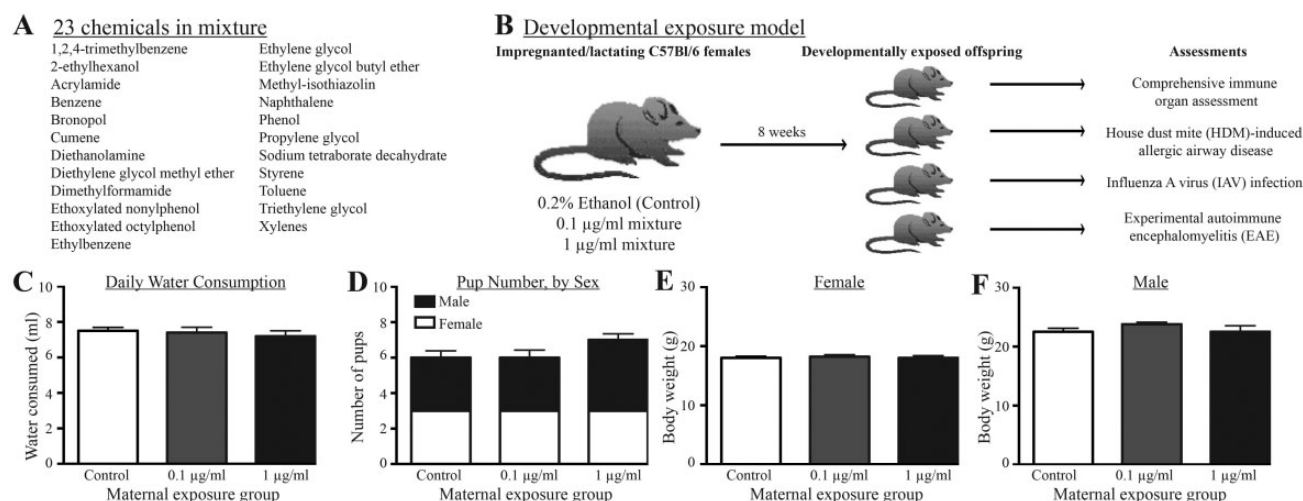


Figure 1. Experimental design and average values for maternal and litter parameters. A, The mixture of 23 chemicals added, in equimass proportions, to the drinking water. B, Pregnant C57Bl/6 dams were placed on control water (0.2% ethanol) or water containing 0.1 or 1 µg/ml of the mixture on day of pregnancy (GD0), through weaning (PND21). There were 10 dams in each group. At maturity (6–8 weeks), offspring of separate dams from each treatment group were randomly assigned to one of four assessment groups for the indicated assessments: immune organ cellularity, HDM-induced airway disease, IAV infection, or EAE. Within each immunological assessment, there were at least 8 nonsibling males and 8 nonsibling females from each developmental exposure group. C, Average daily water consumption by dams. D, Average number of male and female offspring per litter according to treatment group. E, F, Average body weight of adult (8-week) female and male offspring by exposure group. Error bars represent SEM.

(Bar Harbor, ME). Mice were housed in pre-washed polysulfone microisolator cages under specific pathogen-free conditions on a 12 h light/dark cycle. Mice received a standard chow diet (LabDiet 5010, St. Louis, MO), and glass water bottles containing reverse osmosis-purified water were used. Mice were randomly paired, and checked daily for a vaginal plug, indicating pregnancy. On gestational day (GD) 0, dams were separated from sires and randomly placed into one of three groups: control, 0.1 µg/ml, or 1.0 µg/ml of the 23-chemical mixture (Figs. 1A and 1B). Specifically, the dams' drinking water was spiked with an equimass mixture of 23 chemicals (Figure 1A) at a final concentration of 0.1 or 1.0 µg/ml of each constituent chemical (exposed groups), or with 0.2% ethanol (control group). These concentrations in the drinking water result in an estimated 30 and 300 µg/kg body weight/day to the dam, respectively (Kassotis et al., 2015, 2016a). The doses were chosen based on estimates of environmentally relevant oral exposures, such that the two concentrations are similar to levels detected in surface and groundwater in UOG production regions (Cozzarelli et al., 2017; DiGiulio and Jackson, 2016; Gross et al., 2013; Orem et al., 2017; United States Environmental Protection Agency, 2015). Dams remained on treated water from GD0 until pup weaning at post-natal day (PND) 21. Water consumption was monitored daily. The water and water bottles were changed weekly, with freshly prepared dilutions (Kassotis et al., 2015, 2016a). This reduces potential degradation or loss of VOCs to the bottle head-space, diminishing fluctuations in the concentration over time.

After weaning, pups were maintained on unspiked water until sacrifice. Time to parturition, pup number, sex, and body weight were recorded. No culling of litters was performed, and littermates were housed in same-sex groups. Other than determining sex, offspring were randomly assigned to each immunological assessment (Figure 1B). Eight to ten age-matched males, and eight to ten age-matched females from each of the three developmental exposure groups were used in the immunological assessments. Within each exposure group, offspring of the same sex were from different dams, and the age range of the

offspring was uniformly distributed across the groups (6–10 weeks of age). All experiments were initiated in the morning.

All animal treatments and work with infectious agents were conducted with prior approval of Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Rochester. The University has accreditation through the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were treated humanely and with due consideration to alleviation of any distress and discomfort. All guidelines from the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals were followed in handling of vertebrate animals.

Collection and preparation of cells. Cells from mediastinal, peripheral (inguinal, axillary, and brachial), and cervical lymph nodes, thymus, spleen, or bone marrow were collected and processed into a single suspension as described previously (Reilly et al., 2015; Vorderstrasse et al., 2006). Erythrocytes were removed using an ammonium chloride lysing solution. The number of viable cells in each sample was determined using TC10 automated cell counter (Bio-Rad, Hercules, CA) or a hemocytometer and Trypan blue exclusion.

Analytical flow cytometry. Flow cytometry was used to identify and enumerate specific cell populations from offspring that were immunologically naïve, and from offspring that were used in the HDM, IAV, or EAE models. Isolated cells were incubated with previously determined optimal concentrations of fluorochrome-conjugated antibodies. Nonspecific staining was blocked by incubating cells with an anti-mouse CD16/32 mAb. For the work reported herein, the following antibodies against cell-surface antigens were used: CD3ε (clone 145-211), CD4 (clone RM4-5), CD8α (clone 53-6-7), CD11c (clone N418), CD11b (clone M1/70), CD19 (clone 1D3), CD25 (clone PC61.5), CD34 (clone RAM34), CD44 (clone IM7), CD62L (clone MEL-14), CD103 (clone 2E7), CD105 (clone ID4B), CD117 (cKit; clone 2B8), CD127 (clone A7R34), CD150 (clone TC15-12F12.2), F4/80 (clone BM8), gamma delta (γδ) TCR [clone GL3], Gr-1 (clone RB6-8C5), I-Ab

(clone M5/114.15.2), NK1.1 (clone PK136), Sca-1 (clone D7), or a lineage antibody cocktail (CD3, CD11b, CD45R, Ly-G6 [Gr1], and Ter119). To identify virus-specific CD8⁺ T cells, allophycocyanin (APC)-labeled major histocompatibility (MHC) class I tetramers containing an immunodominant peptide epitope of HKx31 (nucleoprotein, D^{NP}_{366–375}) were used. To identify CD4⁺ T cell subsets, after labeling with antibodies against CD4 and CD25, cells were fixed and permeabilized (Foxp3 Staining Kit, eBioscience, San Diego CA), and incubated with fluorochrome-conjugated antibodies against Foxp3 (clone FJK-16S), GATA3 (clone L50-823), ROR γ t (clone Q31-378), and TBet (clone 4I8O), as described previously (Boule et al., 2014). All antibodies were purchased from eBioscience (San Diego, CA) or BD Biosciences (San Jose, CA). Fluorescence minus one (FMO) controls were used to determine non-specific fluorescence and define gating parameters. Data were collected using an LSRII flow cytometer (BD Biosciences, San Jose, CA), and analyzed using the FlowJo software program (TreeStar, Ashland, OR).

House dust mite extract (HDM)-induced allergic airway disease. HDM (Dermatophagoides pteronyssinus) extract (lot #262538, Greer Laboratories, Lenoir, NC) was diluted in sterile PBS. Adult (6–8 weeks of age) female and male offspring from each developmental exposure group were sensitized and challenged by daily administration of 3 μ g HDM intranasally (i.n.) for 10 days, which induces CD4⁺ T cell-dependent allergic airway disease (Knowlden et al., 2016). Forty-eight hours after the last HDM challenge, mice were euthanized, and T cells in the lung-draining mediastinal lymph nodes (MLN) were examined using flow cytometry. Also, bronchoalveolar lavage (BAL) was performed by instilling 0.75 mL PBS twice into the lungs using a Teflon cannula to collect immune cells in airways. BAL and MLN were collected from the same mice, and the BAL was collected first. Differential cell counts of BAL cells were performed after cytocentrifugation onto coded slides, and staining with Hema3 Staining Set (Fisher Scientific, Waltham, MA).

Influenza A virus infection. Male and female offspring (8–10 weeks of age) from each developmental exposure group were anesthetized by intraperitoneal (i.p.) injection of avertin (2,2,2-tribromoethanol), and infected (i.n.) with 120 hemagglutinating units (HAU) of IAV (HKx31; H3N2) diluted in sterile, endotoxin-tested PBS. The virus was prepared and titered as described previously (Warren et al., 2000). Morbidity and mortality were monitored daily, starting on the day of infection, and T cells were examined on the 9th day after infection using flow cytometry.

Induction of experimental autoimmune encephalomyelitis (EAE). To determine whether developmental exposure to chemicals associated with UOG effects a T cell-dependent disease that mirrors aspects of a human autoimmune disease, we used EAE, which models multiple sclerosis (Mendel et al., 1995). Developmentally exposed adult offspring were immunized with an emulsion of myelin oligodendrocyte glycoprotein (MOG_{35–55}), and disease progression was monitored every other day over a 6-week period (Robinson et al., 2014). Adult offspring (6–10 weeks of age) were immunized by subcutaneous injection with an emulsion of the MOG_{35–55} peptide (200 μ g/mouse; AnaSpec, Fremont, CA) and complete Freund's adjuvant (4 mg/mL *M. Tuberculosis*; Becton Dickinson, Franklin Lakes, NJ) at day 0 (Stromnes and Gorman, 2006). Two doses of pertussis toxin (400 ng/mouse; List Biologicals, Campbell, CA) were given intraperitoneally: one on day 0 and the other on day 2 (Stromnes and Gorman, 2006). To identify and enumerate T cell subsets in the cervical lymph

nodes 8–10 female mice and 8–10 male mice from each exposure group were sacrificed on days 7, 21, or 42 after immunization. There were no selection criteria used to determine which mice were sacrificed at a particular point in time after disease was initiated. Using 10 female and 10 male offspring per exposure group, disease progression was monitored and scored every other day for 42 days. Disease symptoms were scored using an established system: 0 = normal mouse, 1 = limp tail, 2 = limp tail and hind limb weakness, 3 = partial hind limb paralysis, 4 = complete hind limb paralysis, 5 = moribund (Stromnes and Gorman, 2006). During disease scoring, information on which exposure group the mice were in was not available (ie, disease scoring was performed in a blinded manner). At each point in time relative to administration of MOG peptide, offspring of the same sex were from a different treated dam.

Statistical analyses. The dam is defined as the statistical unit for all experiments. All offspring in each treatment group and at each point in time relative to immunological assessment were from a different treated dam. Data were analyzed using JMP software (SAS, Cary, NC). Differences between exposure group, sex, and, where applicable, time relative to immune challenge were evaluated using a two-way analysis of variance (ANOVA). Analyses included comparisons within sex across exposure groups, and across sex and exposure groups, using Tukey post-hoc tests, whereas comparisons within sex were analyzed using a Dunnett's post-hoc test, with offspring of vehicle dams as the control group. Survival after infection was analyzed using a Mantel-Cox test. The onset of symptoms in mice with EAE was analyzed using a Kaplan Meier curve, and comparisons between treatment groups were performed using a Wilcoxon test. Differences were considered statistically significant when *p*-values were less than or equal to .05. Error bars on all graphs represent the standard error of the mean (SEM).

RESULTS

Developmental Exposure and Immune System Ontogeny

To determine whether developmental exposure to a mixture of water contaminants that have been associated with UOG has immunological consequences, we exposed pregnant dams to water containing an equimass mixture of 23 chemicals (Figs. 1A and 1B) at two concentrations (0.1 and 1 μ g/mL), or to water spiked with the vehicle control (0.2% ethanol). Dams remained on the treated water until their pups were weaned. There was no difference in daily water consumption across the treatment groups (Figure 1C), and dam body weights were not different across the groups (Supplementary Table 1). Consistent with prior reports using this mixture (Kassotis et al., 2015), there was no difference in pregnancy success or the time to parturition (Supplementary Table 1), number of pups per litter, or sex ratio of pups among treatment groups (Figure 1D). At 8 weeks of age, the body weight of female and male offspring exposed to 0.1 or 1 μ g/mL of the chemical mixture during development was not different than weights of sex- and age-matched offspring of vehicle control-treated dams (Figs. 1E and 1F).

The cellularity of primary and secondary lymphoid organs of male and female offspring that were developmentally exposed to this mixture was compared with age and sex-matched offspring of dams given vehicle control water. Within the same sex, there were no statistically significant differences in the total number of cells recovered from the thymus, spleen, or lymph nodes across the three treatment groups (Table 1). However,

Table 1. The Number of Immune Cells in Immunologically Naïve Female and Male Offspring

	Female 0.1 µg/ml versus Control			Male 0.1 µg/ml versus Control			F versus M			Female 1 µg/ml versus Control			Male 1 µg/ml versus Control			F versus M	
	Control	0.1 µg/ml	p-Value ^a	Control	0.1 µg/ml	p-Value ^a	p-Value ^b			1 µg/ml	p-Value ^a		1 µg/ml	p-Value ^a		p-Value ^b	
Bone marrow																	
Cell count	1243333 ± 742930	1114833 ± 1153077	.53	11850000 ± 640638	14412500 ± 781658	.07	.15			8305000 ± 150000	.05*		14050000 ± 819807	.12		.01*	
HSC	81.7 ± 30.3	123 ± 26	.56	181 ± 47	233 ± 41	.63	.10			277 ± 40	.02*		232 ± 44	.64		.56	
LSK	1681.2 ± 114.3	1681 ± 651	.99	1617 ± 154	1746 ± 190	.24	.84			1249 ± 189	.76		1993 ± 148	.81		.04*	
LK	3727.2 ± 3013	38353 ± 9465	.99	40321 ± 1143	50790 ± 5188	.09	.27			33628 ± 192	.89		50129 ± 2483	.12		.01*	
MPP	1441.3 ± 111.7	1335 ± 565	.97	1253 ± 91	1207 ± 214	.97	.82			745 ± 185	.45		1560 ± 146	.33		.03*	
Lineage ⁻	334137 ± 40143	284293 ± 89951	.81	313340 ± 23271	364988 ± 42847	.48	.41			305411 ± 16724	.94		404781 ± 32400	.15		.12	
CLP	4561 ± 1431	4516 ± 2171	.99	4639 ± 446	4691 ± 778	.98	.93			156 ± 38	.25		4616 ± 163	.99		<.0001*	
GMP	3229.2 ± 1431	4890 ± 444	.10	4417 ± 499	5646 ± 685	.23	.44			3727 ± 514	.76		5258 ± 349	.96		.07	
Pre-GM	8716.2 ± 691.5	8785 ± 2383	.99	11272 ± 316	14981 ± 1787	.09	.09			5484 ± 265	.39		14625 ± 931	.13		.003*	
Pre-MegE	15717 ± 1315	14403 ± 3406	.89	14148 ± 331	17659 ± 1437	.10	.37			14119 ± 255	.88		16469 ± 1344	.31		.31	
Thymus																	
Cell count	134700000 ± 15085534	7992833 ± 38156620	.31	8607500 ± 6312702	84687500 ± 4441583	.97	.89			198750000 ± 33250000	.29		77562500 ± 1852406	.36		.004*	
DN1	1029398 ± 128817	629804 ± 229799	.22	652118 ± 46528	633010 ± 37150	.95	.99			993675 ± 50325	.99		593431 ± 62092	.63		.02*	
DN2	154645 ± 17105	123131 ± 56585	.78	82867 ± 4305	80256 ± 4563	.95	.41			150895 ± 41665	.99		81100 ± 9931	.98		.08	
DN3	1684308 ± 190926	1055676 ± 502342	.36	979296 ± 62343	968785 ± 21799	.97	.84			1995775 ± 324225	.79		933129 ± 14914	.63		.01*	
DN4	929283 ± 44022	1039598 ± 175690	.81	927516 ± 118930	867171 ± 69782	.88	.35			1045175 ± 300425	.83		812128 ± 111851	.65		.40	
DP	64765250 ± 4519003	4214308 ± 21474537	.47	43490400 ± 3354243	43503338 ± 5616368	1	.95			79919750 ± 22160250	.75		41521763 ± 2323515	.92		.05	
DP CD3 ⁺	37535 ± 4244	35116 ± 18488	.98	94271 ± 24827	66288 ± 9805	.45	.17			35117.5 ± 13603	.99		90209 ± 14613	.98		.08	
TCRβ ⁺	6829968 ± 476592	6791068 ± 2675537	.99	6917440 ± 462168	5960451 ± 41223	.34	.73			7544450 ± 1619550	.95		5804218 ± 724325	.25		.30	
CD4 ⁺	5563755 ± 415844	4480870 ± 1974304	.79	4891533 ± 349205	4651036 ± 137026	.82	.92			7347550 ± 1886050	.62		4259121 ± 401819	.31		.08	
CD8 ⁺	680505 ± 55178	930392 ± 96601	.16	885978 ± 206229	751826 ± 106954	.75	.29			533375 ± 185825	.53		723828 ± 105159	.67		.38	
TCRγδ ⁺	483158 ± 45652	313629 ± 138319	.33	378986 ± 27842	377249 ± 23029	.99	.62			526650 ± 30150	.93		395438 ± 69299	.95		.28	
Treg	126705 ± 11419	120334 ± 25422	.97	109801 ± 9453	103152 ± 7950	.80	.49			151058 ± 36863	.69		88345 ± 7749	.18		.07	
Spleen																	
Cell count	86737500 ± 1371298	8221666 ± 31853497	.98	8998750 ± 10669707	107612500 ± 8841907	.32	.06			76000000 ± 19500000	.93		117512500 ± 6614390	.10		.42	
CD19 ⁺	42901875 ± 8244859	4150248 ± 17869820	.99	4446851 ± 10596693	57843813 ± 5629245	.38	.36			42249750 ± 13999750	.99		60730938 ± 9521355	.26		.17	
CD4 ⁺	9179363 ± 1756623	10686967 ± 4435512	.91	11378904 ± 2552025	14578738 ± 865956	.33	.36			7227550 ± 1577550	.88		15235463 ± 969390	.22		.01*	
CD8 ⁺	6755044 ± 1108823	7373400 ± 2819035	.96	7579901 ± 1212533	9894250 ± 747637	.18	.36			4506000 ± 1042550	.68		9921128 ± 642899	.17		.01*	
NK1.1 ⁺	1864925 ± 286698	1721660 ± 705751	.97	2457671 ± 244963	2309921 ± 295240	.86	.43			1148275 ± 255575	.55		2769471 ± 80402	.54		.001*	
TCRγδ ⁺	1404270 ± 224021	1332622 ± 564340	.99	1378785 ± 208629	1592971 ± 114013	.49	.62			1039450 ± 288000	.77		1663444 ± 65032	.31		.04*	
Treg	492888 ± 134031	572553 ± 240016	.93	758630 ± 230462	933366 ± 142882	.67	.23			441275 ± 45775	.97		995236 ± 64601	.5		.01*	
F4/80 ⁺	2736355 ± 362139	2959867 ± 701954	.94	2526675 ± 389486	3384780 ± 384762	.22	.59			3851600 ± 761050	.38		3708730 ± 308728	.08		.84	
Gr-1 ⁺	4945213 ± 691402	5847227 ± 1601634	.79	5473478 ± 1065955	7106785 ± 546481	.26	.44			5581725 ± 960025	.91		7565911 ± 490051	.14		.10	
Gr-1 ⁺ CD11b ⁺	449216 ± 65317	889747 ± 275714	.21	374870 ± 33306	469855 ± 33816	.14	.13			1457325 ± 242575	.02*		542254 ± 34726	.01*		.004*	
CD11c ⁺ MHCII ^{hi}	1196778 ± 203674	1127800 ± 425883	.98	1103168 ± 222118	1405939 ± 137023	.37	.51			915300 ± 163850	.78		1501859 ± 119692	.21		.04*	
CD11b ⁺ DCs	135978 ± 38691	146450 ± 46234	.98	140680 ± 22079	180088 ± 4949	.17	.43			80218 ± 12418	.61		196008 ± 13632	.54		.01*	
CD103 ⁺ DCs	21814 ± 5846	29950 ± 12584	.77	27152 ± 7706	27782 ± 9831	.99	.90			20668 ± 12758	.99		40007 ± 9565	.53		.30	
Lymph nodes																	
Cell count	906250 ± 356587	1246333 ± 230860	.88	1205250 ± 142966	1281250 ± 248510	.95	.92			1750500 ± 1279500	.57		665000 ± 86920	.12		.24	
NK1.1 ⁺	339467 ± 152821	376573 ± 163194	.99	324370 ± 20897	418592 ± 103462	.48	.81			726215 ± 510026	.49		194269 ± 20803	.28		.18	
CD19 ⁺	184994 ± 89183	206919 ± 78508	.99	195106 ± 16840	235362 ± 90192	.82	.83			428606 ± 319805	.45		102250 ± 17897	.41		.17	
TCRγδ ⁺	131612 ± 57008	166186 ± 59364	.95	143302 ± 13848	195615 ± 78622	.66	.37			293009 ± 219062	.46		74033 ± 13529	.49		.24	
CD4 ⁺	3744 ± 1433	4361 ± 1424	.98	4831 ± 845	4884 ± 1376	.99	.83			8238 ± 6306	.45		1909 ± 206	.09		.17	
CD8 ⁺	18441 ± 10365	22114 ± 7900	.95	29839 ± 11946	14580 ± 3132	.27	.79			18404 ± 13411	1		6903 ± 997	.08		.18	
Treg	13911 ± 6572	13545 ± 4531	.99	16110 ± 3418	22151 ± 10075	.73	.52			34468 ± 26132	.39		8232 ± 1871	.59		.18	
F4/80 ⁺	6276 ± 2376	11119 ± 2556	.55	6332 ± 439	7071 ± 773	.61	.14			10887 ± 8202	.65		3314 ± 540	.01*		.21	

Continued

Table 1. (continued)

	Female 0.1 µg/ml versus Control		p-Value ^a	Male 0.1 µg/ml versus Control		F versus M		Female 1 µg/ml versus Control		Male 1 µg/ml versus Control		F versus M
	Control	0.1 µg/ml		Control	0.1 µg/ml	p-Value ^a	p-Value ^b	1 µg/ml	p-Value ^a	1 µg/ml	p-Value ^a	
Gr-1 ⁺	51600 ± 20201	74254 ± 23100	.91	62885 ± 63223	82352 ± 29324	.66	.85	163213 ± 117668	.25	31898 ± 5218	.39	.14
Gr-1 ⁺ CD11b ⁺	624 ± 266	1992 ± 1385	.45	524 ± 85	613 ± 196	.84	.30	1766 ± 871	.63	251 ± 33	.26	.04*
CD11c ⁺ MHCII ^{hi}	7617 ± 3016	11403 ± 3023	.79	7074 ± 193	9223 ± 1931	.38	.56	15758 ± 10906	.47	4117 ± 376	.19	.16
CD11b ⁺ DCs	1993 ± 679	4089 ± 1484	.35	1610 ± 292	2094 ± 144	.18	.17	2521 ± 1721	.93	1120 ± 68	.17	.26
CD103 ⁺ DCs	1729 ± 749	2358 ± 1345	.91	1540 ± 177	1925 ± 613	.69	.76	3302 ± 2455	.66	718 ± 85	.25	.16

The table depicts the mean number ($\pm$ SEM) of the indicated cell population in primary and secondary immune organs from immunologically naïve male and female offspring at maturity (6 weeks of age). There were 4–8 mice of each sex per group, and same-sex offspring within each group were from different dams. Bone marrow, thymus, and peripheral lymph nodes (PLN) were isolated and cell populations identified using multiparametric analytical flow cytometry. Cells enumerated in the bone marrow include: HSC (Lineage[*Lin*]^{neg}Sca-1^{neg}cKit⁺CD105^{hi}CD150^{hi}), LSK cells (Lin^{neg}Sca-1^{neg}cKit⁺CD105^{hi}CD150^{hi}), multipotent progenitors (MPP; Lineage^{neg}Sca-1^{neg}cKit⁺CD105^{hi}CD150^{neg}), common lymphocyte progenitors (CLP; Lineage^{neg}Sca-1^{neg}cKit⁺CD105^{hi}CD150^{neg}CD127⁺), granulocyte monocyte progenitors (GMP; Lin^{neg}cKit⁺CD16/32⁺CD150^{neg}), pre-granulocyte/monocyte cells (pre-GM; Lin^{neg}cKit⁺CD16/32⁺CD150^{neg}CD105^{hi}), pre-megakaryocyte/erythrocyte cells (pre-MegE; Lin^{neg}cKit⁺CD16/32⁺CD150^{neg}CD105^{hi}), pre-megakaryocyte/erythrocyte cells (pre-MegE; Lin^{neg}cKit⁺CD16/32⁺CD150^{neg}CD105^{hi}), DN1 (CD44⁺CD25^{neg}CD4⁺CD8^{neg}), DN2 (CD44⁺CD25^{neg}CD4⁺CD8^{neg}), DN3 (CD44⁺CD25^{neg}CD4⁺CD8^{neg}), DN4 (CD44⁺CD25^{neg}CD4⁺CD8^{neg}), double positive (DP; CD3⁺CD4⁺CD8⁺), CD8 SP (CD3⁺CD4⁺CD8⁺), Treg (Foxp3⁺CD25⁺CD4⁺CD8^{neg}), CD4 SP (CD3⁺CD4⁺CD8^{neg}), CD4⁺ T cells (CD3⁺CD4⁺), Treg (Foxp3⁺CD25⁺CD4⁺), neutrophils (Gr-1⁺), macrophages (F4/80⁺), CD11b⁺ DCs (CD11b⁺CD103⁺MHCII^{hi} cells), CD11c⁺ MHCII^{hi} cells and CD103⁺ DCs (CD11b⁺CD103⁺CD11c⁺MHCII^{hi} cells), $\gamma\delta$ T cells ($\gamma\delta$ TCR⁺CD3⁺CD4⁺CD8⁺), NK cells (CD3⁺CD39⁺NK1.1⁺), B cells (CD3⁺CD19⁺).

^aThe p-value for mixture-exposed group compared with control group within the indicated sex.

³The *p*-value for female versus male offspring from dams given indicated concentration of the mixture.

*Demarcates $p \leq .05$.

there were several differences in the percentage or number of several immune progenitor or lineage committed cell populations in the bone marrow, thymus, spleen, or lymph nodes (Tables 1 and 2). For example, in female offspring of dams given water containing the higher concentration of UOG mixture (1 µg/ml), there were one-third fewer bone marrow cells (Table 1). Yet, the percentage and number of HSCs in the bone marrow of female offspring was significantly elevated in these offspring. The mean percentage and number of HSCs in female offspring of dams given 0.1 µg/ml of the mixture was almost twice that female offspring of control dams, but these differences were not statistically significant. As another example, the percentage of granulocyte monocyte precursors (GMPs) was 1.7- to 1.9-fold higher than control in female offspring of dams exposed to the mixture (Table 2).

Among male offspring, neither GMPs nor other leukocyte lineages in the bone marrow or thymus were different from control in the offspring of dams exposed to either concentration of the mixture (Tables 1 and 2). In peripheral immune organs, there were modest shifts in leukocytes, such as a diminution in the percentage of CD8⁺ T cells in spleens of female offspring of dams given 1 μg/ml (Table 2), and an increase in the number of Gr1⁺CD11b⁺ myeloid cells in the spleen of both male and female offspring of dams treated with the higher concentration (Table 1). In addition to differences in cellularity associated with developmental exposure within sex, there were some differences between male and female offspring with regard to the percentage and number of lineage progenitors and lineage committed cell types in the bone marrow and thymus (eg, LK, pre-GM, DN, DP, CD4 SP, Treg).

HDM-Induced Allergic Airway Disease

Offspring were sensitized and challenged with HDM, which induces CD4⁺ T cell-dependent allergic airway disease (Knowlden *et al.*, 2016). Following HDM challenge, there were no differences in the number of CD4⁺ T cells in the MLN of male or female offspring from all three exposure groups (Supplementary Table 2). However, female offspring of dams given 0.1 µg/mL of the mixture had a statistically significant decrease in the percentage of CD4⁺ T cells. Moreover, additional differences associated with developmental exposure were revealed when subpopulations of CD4⁺ T cells were further examined. Two major subsets of helper CD4⁺ T cells that drive allergic immune responses and contribute to pathology are Th2 cells and Th17 cells (Hirahara and Nakayama, 2016; Vroman *et al.*, 2015). Also, Tregs control the magnitude and duration of the response (Langier *et al.*, 2012). Female mice that were developmentally exposed to 0.1 µg/ml of the mixture, but not 1 µg/ml of the mixture, had a significant increase in the percentage of Th2 cells compared with HDM challenged female offspring of control dams (Figure 2A; Supplementary Table 3). Female offspring exposed to either 0.1 or 1 µg/ml of the chemical mixture also had a greater frequency of Th17 cells (Figure 2C; Supplementary Table 3). Although the frequency of Tregs in female mice that were developmentally exposed to the chemical mixture was not significantly different from control offspring (Figure 2E; Supplementary Tables 2 and 3), the relative proportion of Tregs to Th2 and Th17 cells was diminished (Figs. 2G and 2H). Specifically, the ratio of Treg: Th2 cells was reduced by exposure to 0.1 µg/ml of the chemical mixture, whereas the Treg: Th17 cell ratio was reduced by both doses of the mixture in female offspring (Figs. 2G and 2H). Female offspring that were developmentally exposed to both concentrations of the mixture had a significant increase in airway macrophages (Figure 2I).

Table 2. The Percentage of Immune Cells in Immunologically Naïve Offspring

	Female 0.1 µg/ml versus Control			Male 0.1 µg/ml versus Control			F versus M		Female 1 µg/ml versus Control		Male 1 µg/ml versus Control		F versus M	
	Control	0.1 µg/ml	p-Value ^a	Control	0.1 µg/ml	p-Value ^a	p-Value ^b	1 µg/ml	p-Value ^a	1 µg/ml	p-Value ^a	1 µg/ml	p-Value ^a	
Bone marrow														
HSC	0.0007 ± 0.0002	0.0011 ± 0.0002	.51	0.0015 ± 0.0004	0.0016 ± 0.0002	.96	.07	0.003 ± 0.001	.003*	0.0017 ± 0.0003	.91	.49		
LSK	0.014 ± 0.001	0.014 ± 0.005	.99	0.014 ± 0.002	0.012 ± 0.001	.69	.98	0.015 ± 0.002	.96	0.015 ± 0.002	.92	.97		
LK	0.30 ± 0.02	0.33 ± 0.06	.82	0.34 ± 0.02	0.35 ± 0.03	.95	.37	0.41 ± 0.01	.26	0.36 ± 0.03	.85	.53		
MPP	0.0117 ± 0.0001	0.011 ± 0.004	.98	0.011 ± 0.001	0.008 ± 0.001	.42	.68	0.009 ± 0.002	.79	0.011 ± 0.002	.89	.61		
Lineage ⁻	2.7 ± 0.17	2.4 ± 0.64	.90	2.7 ± 0.28	2.5 ± 0.24	.84	.55	3.68 ± 0.13	.31	2.9 ± 0.12	.76	.15		
CLP	0.04 ± 0.01	0.04 ± 0.02	.99	0.039 ± 0.004	0.03 ± 0.01	.59	.77	0.002 ± 0.001	.25	0.033 ± 0.003	.58	.01*		
GMP	0.026 ± 0.004	0.044 ± 0.001	.03*	0.04 ± 0.01	0.04 ± 0.01	.96	1.0	0.05 ± 0.01	.04*	0.038 ± 0.002	.99	.30		
Pre-GM	0.070 ± 0.003	0.08 ± 0.02	.88	0.09 ± 0.01	0.10 ± 0.01	.80	.09	0.066 ± 0.002	.95	0.11 ± 0.01	.69	.08		
Pre-MegE	0.13 ± 0.01	0.13 ± 0.02	.99	0.120 ± 0.004	0.12 ± 0.01	.95	.55	0.1700 ± 0.00	.19	0.12 ± 0.01	.95	.03*		
Thymus														
DN1	0.76 ± 0.02	1.6 ± 0.86	.41	0.77 ± 0.09	0.76 ± 0.07	.98	.30	0.51 ± 0.06	.93	0.77 ± 0.08	.99	.07		
DN2	0.12 ± 0.01	0.17 ± 0.02	.01*	0.09 ± 0.01	0.09 ± 0.01	.97	.003*	0.07 ± 0.01	.06	0.11 ± 0.01	.86	.47		
DN3	1.25 ± 0.062	1.3 ± 0.09	.99	1.14 ± 0.039	1.15 ± 0.040	.99	.33	1.01 ± 0.005	.06	1.21 ± 0.033	.43	.18		
DN4	0.72 ± 0.09	5.9 ± 4.9	.36	1.1 ± 0.21	1.04 ± 0.139	.95	.29	0.52 ± 0.07	.99	1.04 ± 0.144	.95	.06		
DP	49.2 ± 3.67	37.6 ± 16.1	.62	50.5 ± 1.62	50.9 ± 4.31	.99	.44	39.5 ± 4.55	.76	53.7 ± 3.35	.73	.08		
DP CD3 ⁺	0.03 ± 0.01	0.03 ± 0.01	.85	0.12 ± 0.04	0.08 ± 0.01	.53	.06	0.017 ± 0.004	.49	0.12 ± 0.02	.99	.01*		
TCRβ ⁺	5.3 ± 0.67	14.6 ± 6.4	.19	8.2 ± 0.98	7.1 ± 0.43	.54	.22	3.8 ± 0.19	.95	7.5 ± 0.86	.73	.04*		
CD4 ⁺	4.2 ± 0.27	7.8 ± 2.4	.17	5.7 ± 0.48	5.6 ± 0.39	.94	.32	3.6 ± 0.34	.95	5.5 ± 0.51	.9	.07		
CD8 ⁺	0.54 ± 0.09	0.26 ± 0.05	.36	1.1 ± 0.33	0.91 ± 0.18	.83	.30	0.26 ± 0.05	.99	0.93 ± 0.14	.86	.02*		
TCRγδ ⁺	0.36 ± 0.02	0.55 ± 0.17	.34	0.45 ± 0.05	0.45 ± 0.05	.99	.55	0.27 ± 0.03	.78	0.51 ± 0.08	.73	.04*		
Treg	0.095 ± 0.004	0.63 ± 0.51	.37	0.13 ± 0.02	0.12 ± 0.02	.94	.29	0.08 ± 0.01	.99	0.11 ± 0.01	.66	.06		
Spleen														
CD19 ⁺	48.3 ± 3.16	49.1 ± 3.45	.98	47.2 ± 8.41	53.6 ± 1.19	.59	.22	54.5 ± 4.45	.47	51.5 ± 1.61	.77	.47		
CD4 ⁺	10.8 ± 1.51	12.3 ± 0.984	.66	12.2 ± 1.69	13.7 ± 0.492	.51	.23	9.6 ± 0.39	.81	12.9 ± 0.17	.81	.001*		
CD8 ⁺	7.8 ± 0.39	9.03 ± 0.087	.06	8.3 ± 0.47	9.2 ± 0.19	.09	.49	5.9 ± 0.16	.02*	8.4 ± 0.09	.92	.0001*		
NK1.1 ⁺	2.2 ± 0.18	1.9 ± 0.24	.62	2.8 ± 0.44	2.1 ± 0.14	.16	.55	1.5 ± 0.06	.13	2.4 ± 0.09	.41	.004*		
TCRγδ ⁺	1.6 ± 0.07	1.5 ± 0.11	.72	1.5 ± 0.06	1.49 ± 0.025	.92	.62	1.36 ± 0.030	.16	1.4 ± 0.08	.49	.61		
Treg	0.59 ± 0.14	0.71 ± 0.06	.69	0.78 ± 0.20	0.86 ± 0.07	.89	.21	0.61 ± 0.09	.99	0.85 ± 0.04	.91	.04*		
F4/80 ⁺	3.3 ± 0.36	4.9 ± 1.9	.49	2.8 ± 0.23	3.1 ± 0.13	.29	.30	5.2 ± 0.32	.50	3.1 ± 0.11	.25	.002*		
Gr-1 ⁺	5.8 ± 0.31	8.8 ± 2.2	.24	5.9 ± 0.61	6.6 ± 0.20	.33	.31	7.5 ± 0.67	.63	6.4 ± 0.07	.52	.06		
Gr-1 ⁺ CD11b ⁺	0.53 ± 0.05	2.56 ± 2.01	.38	0.42 ± 0.02	0.44 ± 0.02	.82	.26	1.9 ± 0.19	.66	0.46 ± 0.03	.41	.0002*		
CD11c ⁺ MHCII ^{hi}	1.37 ± 0.044	1.38 ± 0.035	.98	1.2 ± 0.19	1.3 ± 0.07	.77	.42	1.2 ± 0.10	.23	1.3 ± 0.09	.86	.74		
CD11b ⁺ DCs	0.15 ± 0.03	0.20 ± 0.05	.39	0.16 ± 0.02	0.17 ± 0.01	.63	.32	0.11 ± 0.01	.51	0.17 ± 0.01	.72	.03*		
CD103 ⁺ DCs	0.025 ± 0.004	0.04 ± 0.01	.24	0.03 ± 0.01	0.02 ± 0.01	.81	.22	0.02 ± 0.01	.99	0.03 ± 0.01	.97	.51		
Lymph nodes														
NK1.1 ⁺	0.44 ± 0.05	0.34 ± 0.06	.38	0.41 ± 0.07	0.36 ± 0.05	.51	.75	0.45 ± 0.04	.99	0.29 ± 0.03	.86	.04*		
CD19 ⁺	34.80 ± 6.010	28.6 ± 9.17	.77	27.4 ± 1.69	32.3 ± 5.24	.99	.73	43.4 ± 2.55	.68	29.6 ± 1.56	.89	.01*		
TCRγδ ⁺	1.8 ± 0.31	1.7 ± 0.29	.96	2.3 ± 0.70	1.1 ± 0.04	.90	.08	1.06 ± 0.005	.28	1.04 ± 0.068	.85	.87		
CD4 ⁺	19.7 ± 2.56	16.3 ± 5.48	.76	16.5 ± 1.43	16.6 ± 3.62	.79	.97	23.9 ± 0.800	.70	15.2 ± 1.36	.27	.01*		
CD8 ⁺	14.6 ± 1.51	13.2 ± 4.18	.91	12.4 ± 1.98	13.6 ± 3.16	.13	.94	16.3 ± 0.600	.88	10.9 ± 0.959	.11	.02*		
Treg	1.5 ± 0.13	1.1 ± 0.38	.49	1.4 ± 0.37	1.5 ± 0.45	.99	.57	1.9 ± 0.12	.46	1.2 ± 0.18	.89	.07		
F4/80 ⁺	0.72 ± 0.11	0.92 ± 0.23	.60	0.55 ± 0.07	0.55 ± 0.07	.86	.20	0.60 ± 0.03	.85	0.51 ± 0.06	.89	.36		
Gr-1 ⁺	5.9 ± 0.68	5.9 ± 1.4	.99	5.4 ± 0.72	5.8 ± 1.1	.91	.95	9.5 ± 0.20	.09	4.7 ± 0.32	.8	.001*		
Gr-1 ⁺ CD11b ⁺	0.07 ± 0.01	0.18 ± 0.13	.55	0.05 ± 0.01	0.05 ± 0.01	.99	.29	0.14 ± 0.05	.81	0.038 ± 0.003	.77	.03*		

Continued

Table 2. (continued)

	Female 0.1 µg/ml versus Control		Male 0.1 µg/ml versus Control		F versus M		Female 1 µg/ml versus Control		Male 1 µg/ml versus Control		F versus M
	Control	0.1 µg/ml	Control	0.1 µg/ml	p-Value ^b	p-Value ^a	1 µg/ml	p-Value ^a	1 µg/ml	p-Value ^a	p-Value ^b
CD11c ⁺ MHCII ^{hi}	0.85 ± 0.18	0.89 ± 0.07	.97	0.61 ± 0.06	0.71 ± 0.08	.41	.16	0.86 ± 0.08	0.63 ± 0.04	.94	.02*
CD11b ⁺ DCs	0.23 ± 0.04	0.34 ± 0.14	.57	0.14 ± 0.03	0.18 ± 0.03	.53	.26	0.16 ± 0.02	0.18 ± 0.03	.53	.62
CD103 ⁺ DCs	0.19 ± 0.05	0.17 ± 0.07	.95	0.13 ± 0.02	0.14 ± 0.03	.95	.67	0.19 ± 0.01	0.11 ± 0.01	.69	.01*

[illegible]^aThe *p*-value for mixture-exposed group compared with control group within the indicated sex.

^bThe *p*-value for female versus male offspring from dams treated with the indicated concentration of the mixture.

*Denotes $p \leq .05$.

and offspring of dams given 0.1 µg/ml also exhibited increased eosinophils and lymphocytes in the airways after HDM challenge, compared with offspring of control dams (Figure 2I).

In response to HDM sensitization and challenge, the male offspring did not exhibit statistically significant differences in the frequency of Th2 cells, Th17 cells, or Tregs as a result of developmental exposure to this mixture (Figs. 2B, 2D, and 2F). Likewise, male offspring did not present a significant change in Treg: conventional CD4⁺ T cell ratios, although the ratio of Treg:Th2 cells in male offspring exposed to 0.1 µg/m was slightly lower than that of control male offspring (Figs. 2J and 2K). Also in contrast to the female offspring, developmental exposure to this mixture did not change the number of immune cells in airways of male offspring (Figure 2L).

IAV Infection

To assess whether developmental metal exposure changes T cell responses to infection, we administered an IAV challenge that causes mild infection. A sub-lethal infection was selected for two reasons: (1) it better mirrors human IAV infections, because the global burden associated with IAV stems less from mortality and more from the consequences of infection-related illness which range from mild to severe; and (2) it permits measurement of the peak T cell response to infection, which occurs at about day 9 (Boule *et al.*, 2014; Lawrence *et al.*, 2006). The magnitude of key T cell responses generally predicts the overall outcome of the disease (Hayden *et al.*, 1998; Kaiser *et al.*, 2001). Within the same sex, there were no significant differences in the number or percentage of total CD4⁺ or CD8⁺ T cells 9 days after infection based on developmental exposure (Supplementary Tables 4 and 5). Yet, the percentage of viral nucleoprotein (NP)-specific CD8⁺ T cells was significantly elevated in female mice that were developmentally exposed to 1 µg/ml (but not 0.1 µg/ml) of the chemical mixture, compared with female offspring of control dams (Figure 3A). There was also a 1.7-fold increase in the number and percentage of CD44^{hi}CD62L^{lo}CD8⁺ T cells (cytotoxic T lymphocytes, CTL) in female offspring exposed to 1 µg/mL of the mixture; however, this was not statistically significant from CTL frequencies among female offspring of control dams (Supplementary Tables 4 and 5). When the percentage and number of virus-specific CD8⁺ T cells were compared by sex, there was a significant difference between the frequency of NP⁺CD8⁺ T cells in female and male mice from control dams. However, among male offspring in the three exposure groups, there were no significant differences in the percentage (Figure 3B) or number of virus-specific CD8⁺ T cells (Supplementary Table 4), or in the percentage and number of CTL after IAV infection (Supplementary Tables 4 and 5).

CD4⁺ T cells foster the development of a more robust CD8⁺ T cell response to IAV (Kohlmeier and Woodland, 2009; Strutt *et al.*, 2013; Swain *et al.*, 2012). Two critical effector CD4⁺ T cell populations in acute primary IAV infection are Th1 cells and Tregs (Strutt *et al.*, 2013; Swain *et al.*, 2012). These CD4⁺ T cell subsets were enumerated at day 9-post infection in female and male offspring. Compared with offspring of control dams, neither the percentage (Figs. 3C and 3D) nor the number (Supplementary Table 4) of Th1 cells were significantly altered by developmental exposure to either concentration of this mixture in male or female offspring. Similarly, the frequency of Tregs was not affected by developmental exposure to this mixture (Figs. 3E and 3F), nor were there statistically significant differences in the Treg:Th1, Treg:Th17, or Treg:NP⁺CD8⁺ cell ratios in male or female developmentally exposed offspring (Figs. 3G–I). Also, there was no statistically significant diminution in

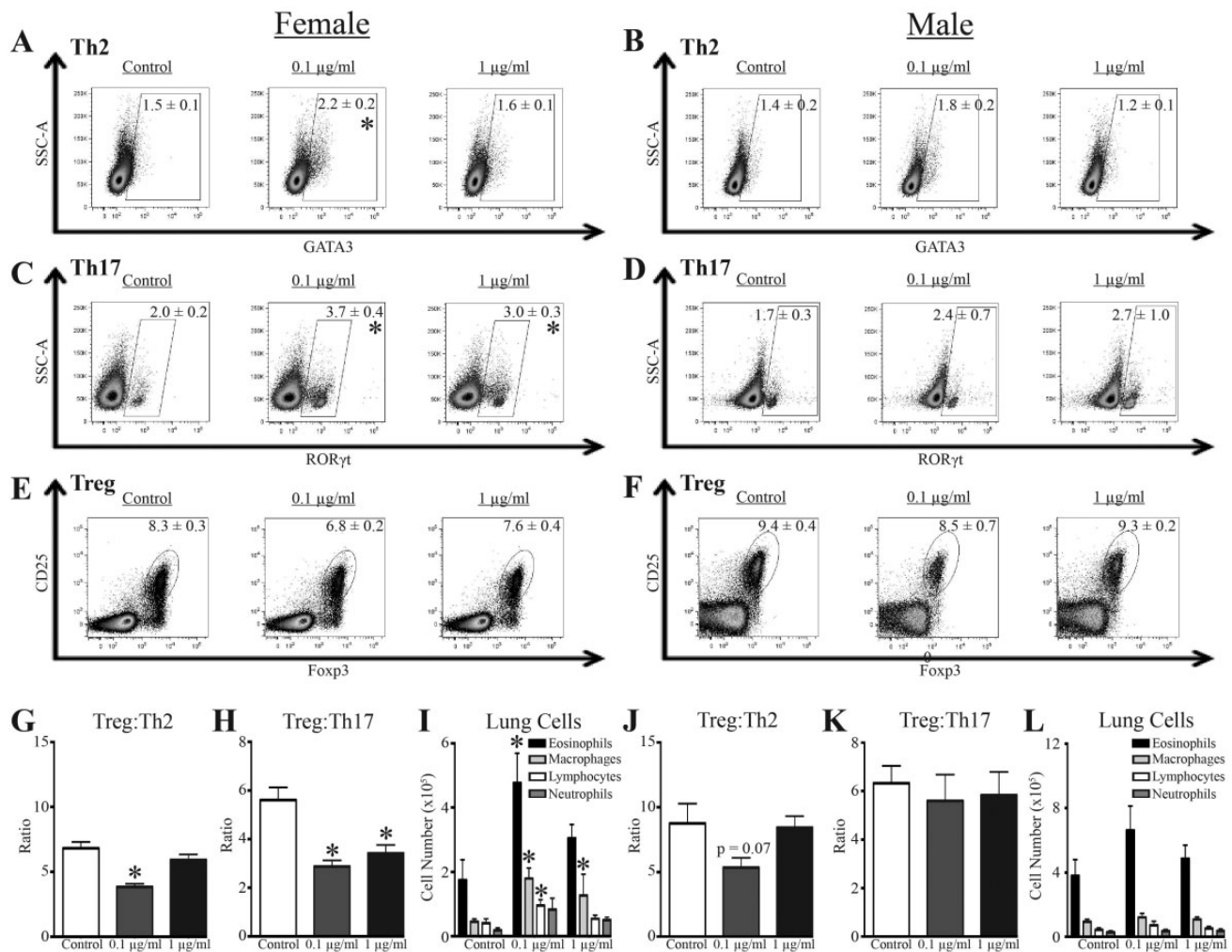


Figure 2. Immunological effects of developmental exposure in a model of allergic airway disease. At maturity (6–8 weeks of age), 9–10 female and 9–10 male offspring from each developmental exposure group were sensitized and challenged with HDM. Within each group, offspring of the same sex were from different dams. A–F, Representative dot plots from flow cytometric analyses of CD4⁺ T cell subsets from MLN 48 h after HDM challenge, and mean percentages ± SEM are depicted according to sex and treatment group for Th2 cells (A–B, GATA3⁺CD4⁺ T cells), Th17 cells (C–D, RORγt⁺CD4⁺ T cells), and Tregs (E–F, Foxp3⁺CD25⁺CD4⁺ T cells). All dot plots are gated on CD4⁺ T cells. G–H, Mean ratio (±SEM) of Treg:Th2 cells and Treg:Th17 cells in MLN from female offspring. I, Mean number (±SEM) of eosinophils, macrophages, lymphocytes, and neutrophils in BAL from female offspring. J–K, Mean ratio (±SEM) of Treg:Th2 cells and Treg:Th17 cells in MLN from male offspring. L, Mean number (±SEM) of eosinophils, macrophages, lymphocytes, and neutrophils in BAL from male offspring. An * represents a p-value < .05 compared with same sex control.

morbidity among female and male offspring that were developmentally exposed to the 23-chemical mixture and age-matched offspring of control dams (Figs. 3M and 3N). Similarly, there were no statistically significant differences in survival among infected offspring from the three developmental exposure groups (Supplementary Figure 1).

Experimental Autoimmune Encephalomyelitis (EAE)

To determine whether developmental exposure to UOG chemical mixture affects a T cell-dependent disease that mirrors aspects of a human autoimmune disease, we used EAE, which models multiple sclerosis (Mendel et al., 1995). The main conventional helper T cells that drive immunopathology during EAE are Th17 and Th1 cells; whereas, Tregs are important in dampening immunopathology caused by these conventional Th subsets (Fletcher et al., 2010). In particular, during EAE, the relative proportion of these two T helper subsets and Tregs influence disease progression. The ratio of Tregs to conventional Th1 and Th17 cells in female (Figs. 4A and 4C) and male (Figs. 4B

and 4D) offspring of dams treated with the mixture were similar to ratios in offspring of control dams. In female mice developmentally exposed to 0.1 µg/ml of the chemical mixture, there were more Th1 cells compared with offspring of control dams, and an overall decrease in the Treg: Th1 ratio (Figure 4A; Supplementary Table 6). In female offspring exposed to the lower dose, there was an increase in the Treg: Th17 ratio, reflecting that there were fewer Th17 cells compared with Tregs (Figure 4C, and Supplementary Table 6). Male developmentally exposed offspring had no significant differences in the ratio of Treg:conventional CD4⁺ T cells, compared with control-exposed mice at all time points (Figs. 4B and 4D). Thus, developmental exposure to chemicals associated with UOG may elicit transient, subtle shifts in CD4⁺ T cell sub-populations during the early onset of EAE that are more prominent in female offspring.

In contrast, there were more pronounced differences in disease severity, time of onset, and progression in female offspring exposed maternally to the chemical mixture. Compared with

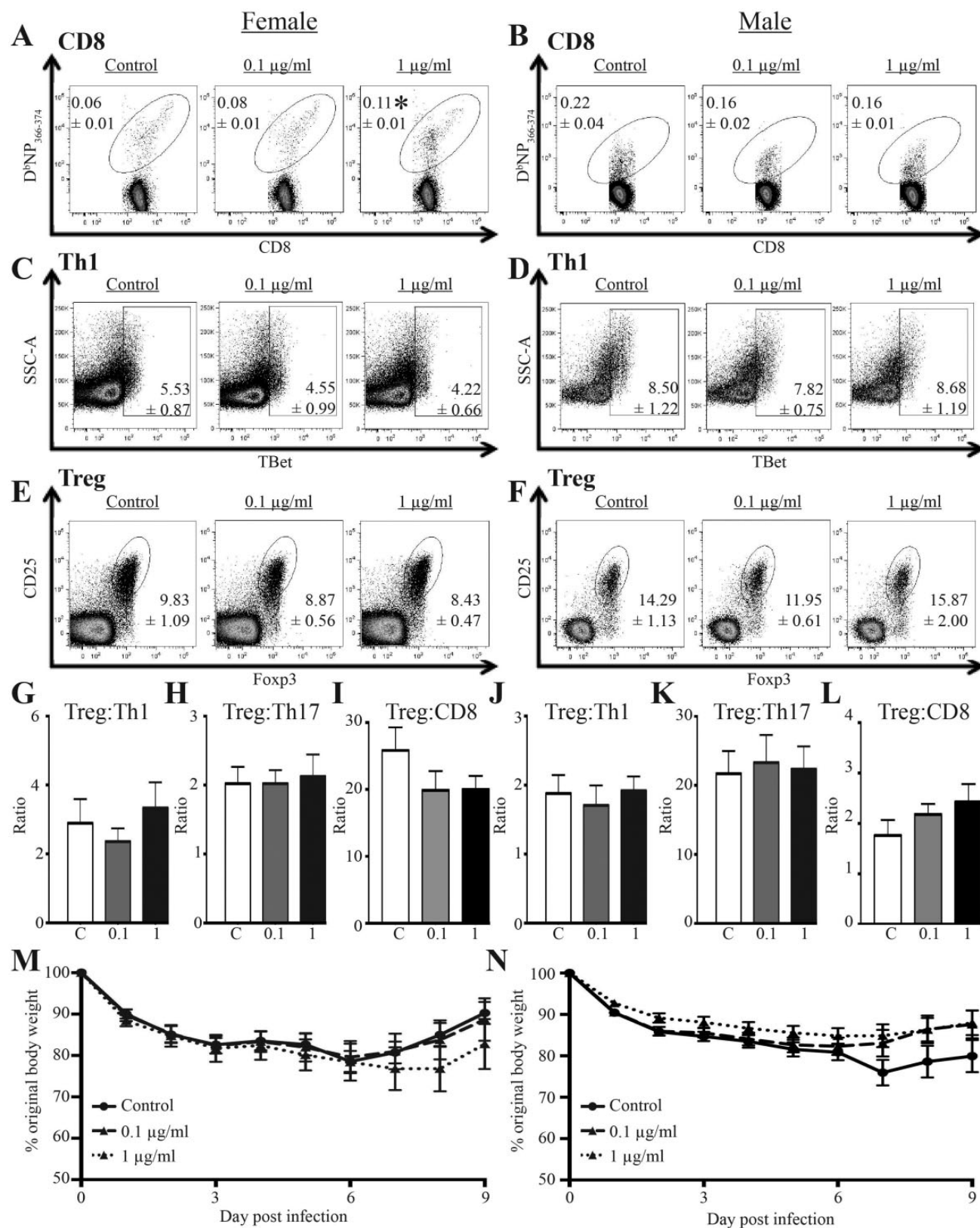


Figure 3. Effects of developmental exposure to chemicals associated with UOG on T cells and body weight change after viral infection. At maturity (6–8 weeks of age), 9–10 female and 9–10 male offspring from each exposure group infected with IAV. Within each group, offspring of the same sex were from different dams. A–F, Representative dot plots depict flow cytometric analyses of T cell subsets from MLN 9 days after infection: IAV NP-specific CD8⁺ T cells (A–B, D^bNP₃₆₆₋₃₇₅⁺CD8⁺ T cells, gated on CD3⁺CD4⁺ cells), Th1 cells (C–D, TBet⁺CD4⁺ cells, gated on CD3⁺CD4⁺ cells), and Tregs (E–F, Foxp3⁺CD25⁺CD4⁺ cells, gated on CD3⁺CD4⁺ cells). The mean percentages (±SEM) of the indicated T cell sub-types in each exposure group and for both sexes are denoted on the plots. G–L, The mean ratios (±SEM) for Treg:Th1 cells, Treg:Th17 cells, and Treg:NP-specific CD8⁺ T cells for each exposure group and separated by sex. M–N, Mean (±SEM) body weight change following infection for female (M) and male (N) offspring. An * represents a p-value ≤ .05 compared with sex matched offspring of control dams.

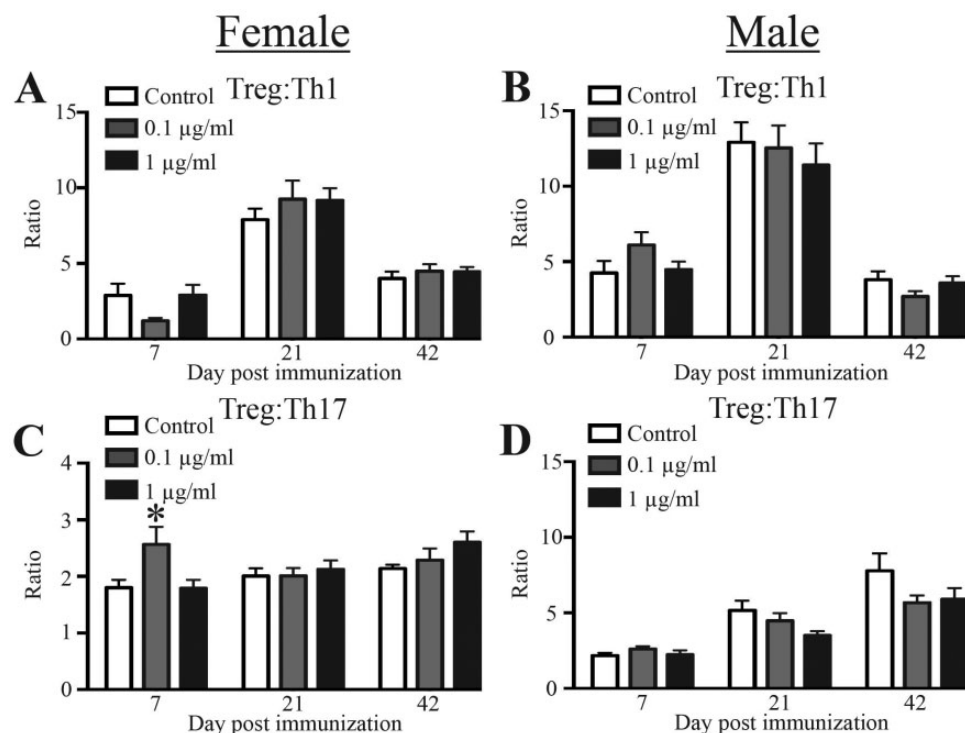


Figure 4. CD4⁺ T cell subset proportions during EAE disease progression. Twenty-six adult (6–8 weeks of age) female and male offspring from each exposure group were immunized with a CFA/MOG_{35–55} emulsion to induce EAE. To enumerate CD4⁺ T cells in cervical lymph nodes, 8–10 female and 8–10 male mice from each group were sacrificed 7, 21, or 42 days after immunization. Mice were randomly assigned to each time point. A–D, The bar graphs depict the mean Treg:Th1 ratios (A–B) and mean Treg:Th17 ratios (±SEM) (C–D). Same sex offspring at each timepoint are from different dams. An * represents a p-value ≤ .05 compared with same sex control on the same day post immunization.

the female offspring of control dams, disease onset was expedited in female offspring exposed to 0.1 and 1 µg/ml of the chemical mixture during development (Figure 5A). On an average, disease onset in female offspring of dams treated with either dose of the chemical mixture occurred about 3–4 days earlier than in their untreated counterparts (Figure 5B). Female offspring that were developmentally exposed to 0.1 µg/ml, but not 1 µg/ml, also had significantly higher disease scores over time compared with control offspring (Figure 5C). Developmentally exposed male offspring, on the other hand, showed no significant difference in the onset, progression, or severity of disease symptoms when compared with control offspring (Figs. 5D–F).

DISCUSSION

Contamination of water supplies is a major global environmental health concern. In particular, threats to water quality due to anthropogenic activities and pollution by chemicals are emergent concerns. However, causality between adverse health outcomes and chemical contaminants in water is challenging to demonstrate. Reasons for this are multifaceted but include that the negative health impacts of chemical exposures often occur in a delayed manner, water testing is inconsistent between regions, and the list of chemicals tested is often incomplete. Further limitations in our ability to understand causal relationships stem from numerous gaps in knowledge of what cell types and physiological functions are perturbed by developmental exposures to water contaminants. The work reported herein establishes that developmental exposure to a mixture of chemicals used in UOG operations leads to several changes in the

cellular composition of the mammalian immune system, and affects T cell composition and function in different disease models. Notably, developmental exposure expedited and exacerbated EAE disease symptoms in female but not male offspring. These results suggest that developmental exposure to chemicals associated with UOG operations has the potential to cause long-lasting, and possibly sex-biased effects on the immune system.

There is scant information on potential developmental immunotoxicity for most of the compounds in this mixture. Moreover, for many constituents of this mixture, there are either no data or the existing evidence of possible immune effects is lean. Nonetheless, there is some evidence that exposure to several components in this mixture, either singly or in smaller groupings, affects the immune system. In particular, benzene and styrene are considered strongly or moderately toxic to the mammalian immune system, respectively (Veraldi et al., 2006). Although the immunotoxicity of benzene has been known for decades, the association of benzene exposure with leukemogenesis and other cancers reflect the best-known aspects of its immunotoxicity (Wang et al., 2012). There are also data demonstrating that the immune system is a target organ of the combination of benzene, toluene, ethylbenzene, and xylenes (BTEX; Bahadar et al., 2014; Bolden et al., 2015). Yet, much of these data focus on cancer, leaving the noncarcinogenic effects of BTEX less well characterized, including its potential developmental immunotoxicity. A recent study reported that inhalation exposure of male mice to a different combination of volatile organics, a mixture of formaldehyde, benzene, toluene, and xylene, decreased the number of T cells in peripheral immune organs (Wang et al., 2016). Other studies have shown that direct

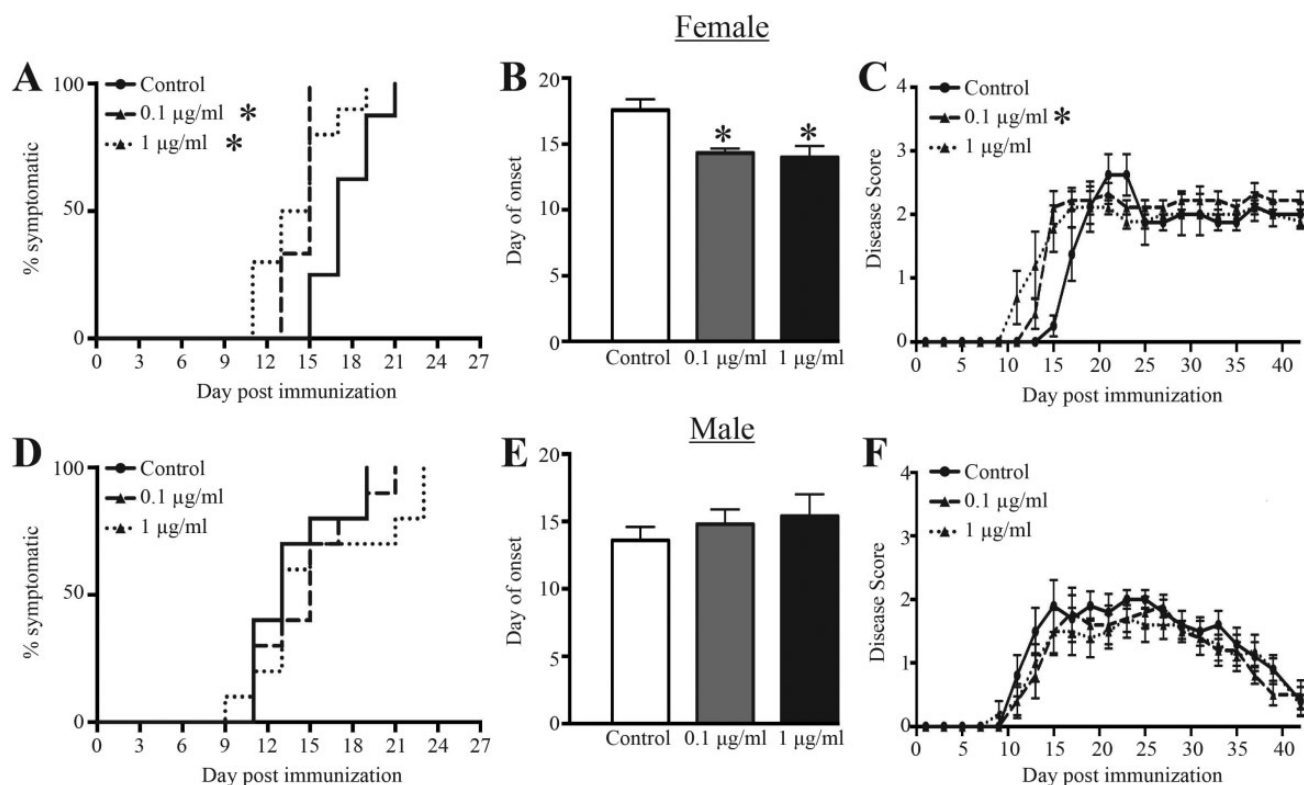


Figure 5. EAE symptom onset and severity following immunization with MOG peptide. Ten adult (6–8 weeks of age) female and male offspring from each exposure group were immunized with a CFA/MOG_{35–55} emulsion to induce EAE. Disease progression was monitored and scored every other day for 42 days. A, D, Kaplan Meier plots show the day of disease onset (disease score ≥ 1) in female (A) and male (D) offspring. B, E, The bar graphs depict the average day of onset for female (B) and male (E) developmentally exposed offspring. C, F, The average EAE disease score ($\pm$ SEM) according to treatment group and day post immunization in female (C) and male (F) offspring. Disease scores were 0 = normal mouse, 1 = limp tail, 2 = limp tail and hind limb weakness, 3 = partial hind limb paralysis, 4 = complete hind limb paralysis, 5 = moribund. An * represents a p-value $\leq .05$ compared with same sex control offspring.

exposure to other volatile organics, including ethylbenzene, is associated with changes in lymphocyte populations and multiple chemical sensitivity (Baines et al., 2004). Thus, although information on immunological effects of some constituents of the 23-chemical mixture is limited or nonexistent, there is evidence that several chemicals within this mixture likely affect the developing immune system.

Among the outcomes that were affected by developmental exposure to this mixture, one of the most evident changes was the advanced time of onset and severity of EAE, particularly in female offspring. This observation, and other data from these mice, suggest that there may be some sex-biased differences. Although a systematic and complete understanding of sex-specific differences in immune responses has not yet been achieved, the endocrine system influences the immune system (Gabriel and Arck, 2014; Oertelt-Prigione, 2012). There is extensive evidence that the immune responses of males and females are inherently different, and that sex affects the timing, magnitude or penetrance of many diseases, including allergic inflammation/asthma, the response to respiratory infections and autoimmune diseases (McClelland and Smith, 2011; Ngo et al., 2014). For example, differences in the frequency of T cells in male compared with female mice infected with IAV have been described previously (Gabriel and Arck, 2014; Oertelt-Prigione, 2012). Furthermore, there is mounting evidence that EDCs influence the immune system during development, and thereby contribute to disease at later stages in life (Kopras et al., 2014; Schug et al., 2011). For instance, developmental exposure to

atrazine, bisphenol A, cadmium, and perfluorooctane sulfonate (separately) leads to sex-based differences in myriad immune system metrics in the offspring (Bauer et al., 2012; Bodin et al., 2014; Boule et al., 2015a,b; Boule and Lawrence, 2016; Hanson et al., 2012; Keil et al., 2008; Ng et al., 2006; O'Brien et al., 2014; Rooney et al., 2003). Conversely, there are examples in which developmental exposure has similar effects on the immune system of both male and female offspring (Mustafa et al., 2011; Roy et al., 2012; Vorderstrasse et al., 2006). There are also cases in which the same agent gives different results across model systems. For example, in two studies using the same mouse strain, maternal dosing design, and dosage, developmental exposure to BPA showed evidence of sex-biased differences in a mouse model of allergic airway diseases, but not in offspring infected with IAV (Bauer et al., 2012; Roy et al., 2012). Collectively, these studies illustrate that the relative sensitivity of one sex or the other to perturbation by a developmental immunotoxicant is multifactorial and includes aspects of the antigenic challenge or injury. Therefore, although the findings of this current study suggest that females may be more sensitive to early life exposure to this mixture, it is premature to conclude firmly that one sex is overall more sensitive to developmental exposure to chemicals associated with UOG.

In addition to differences in which offspring of different sexes exhibited different outcomes, some immune changes showed evidence of dose-responsiveness; however, other effects of developmental exposure were observed at the lower, but not the higher maternal dose. Examples of immune

endpoints that were affected in both the lower and higher dose groups include the Treg:Th17 cell ratio in HDM challenge and the timing of onset in EAE. Yet, other findings show similarities with prior reports of nonmonotonic dose-response relationships in some consequences of exposure to EDCs with offspring of dams given the lower mixture concentration exhibiting a greater effect than higher dose group (Bodin et al., 2013; Vandenberg et al., 2012). Moreover, not all effects showed evidence of nonmonotonicity. For example, EAE disease onset was faster in female offspring of dams exposed to both concentrations of the mixture compared with female controls, whereas the overall disease severity scores were only significantly higher than control in female offspring exposed to the lower concentration. This may reflect dose-dependent differences in target tissues. For example, we measured CD4⁺ T cell populations in peripheral lymph nodes. Cellular changes at the site of the immune response (eg, the CNS) may be different from responses detected in lymphoid tissues. It is also possible that the complex interplay of signaling from CNS-resident cells and CNS-infiltrating immune cells underlies disease onset rate and disease severity, and these events have differential dose sensitivities (Alvarez et al., 2015; Kroner et al., 2009; Lee et al., 2012; Polfiet et al., 2002). Other studies that have examined exposure to some constituents of the mixture suggest that there are direct, dose-dependent alterations in the endocrine and neurological systems, which could trigger or synergize with altered immune function (Bahadar et al., 2015; Kajta and Wojtowicz, 2013). Consequently, developmental exposure to chemicals associated with UOG that have known endocrine-disrupting characteristics, such as the representative mixture used here, may cause persistent changes in the interplay between the immune, nervous, and endocrine systems.

Another finding was the association of developmental exposure to this mixture and changes in T cells after an immune challenge. Developmental exposure significantly shifted the proportion of specific T cell subsets in female offspring in the HDM model, and to a lesser extent in offspring challenged with IAV. This suggests that developmental exposure to this mixture may not affect T cells globally, but impinges on the pathways that are important during T cell responses to challenge. For example, the balance of regulatory and effector T cell subsets is an important indicator of the progression and severity of diseases, including allergic asthma and infections (Chapman and Georas, 2014). Although no studies to date have examined whether living near or working at UOG operations is associated with enhanced respiratory infections, a recent report linked proximity to UOG operations and increased asthma exacerbations (Rasmussen et al., 2016). Thus, examining human T cell subset distribution and responsive capacity may accelerate research in exposed populations in order to define associations between proximity to UOG operations, water contaminants, and altered immune function later in life. Further support for using differentiation of peripheral T cells to evaluate potential immunotoxicants comes from studies of other developmental exposures (reviewed in Boule and Lawrence, 2016). For example, in B6C3F1 mice, maternal exposure to cigarette smoke, another complex mixture, modulates T cell proliferative capacity and dampens their ability to kill tumor cells (Ng et al., 2006; Ng and Zelikoff, 2008). Yet, in other studies the magnitude and direction of change depends upon the anatomical site examined, such as in studies reporting alterations in the proportion of Tregs following developmental exposure to cadmium (Hanson et al., 2012) and dioxin (Boule et al., 2014, 2015b). Thus, although T cells are commonly affected by developmental immunotoxicants, the

consequences measured later in life are highly dependent upon context, including anatomical site, timing, and the profile of T cells that respond to a particular antigenic challenge.

Although we report that developmental exposure to chemicals used in UOG operation has significant effects on the immune system, there are some limitations to our study. For instance, we deliberately selected a dose and strain of IAV that causes mild infection so that mice would survive, clear the virus, and T cell responses could be examined as the infection was resolved. The lack of significant change in morbidity and mortality following IAV infection suggests that developmental exposure to water containing this mixture of 23 chemicals did not overtly compromise aspects of immune function crucial for surviving mild acute respiratory viral infection. Further evaluation using more pathogenic strains of IAV, and other types of viruses, is needed. Also, in all three disease models, we defined CD4⁺ T cell subsets by the expression of lineage-specific transcription factors (Yamane and Paul, 2013), but future studies will be needed to determine exactly how T cell effector function was affected (eg, production of cytokines or other mediators). Also, in assessing immune responses, there are many other cell types that could be examined. It seems possible that the function of additional immune cell types could be altered by developmental exposure to this mixture, which could be productive areas of future research. Finally, environmental exposures may encompass maternal and direct exposures after birth (Boverhof et al., 2014; Dietert and Zelikoff, 2008). The goal of the present study was to characterize whether developmental exposure changed how the immune system responds later in life; hence, only vertical exposure to this mixture was used. Thus, it remains possible that these chemicals cause immune effects that are repaired during the gap between developmental exposure and immune assessment. For example, the immunomodulatory actions of other agents, such as irradiation and anticancer drugs, are not only immediate, but also cause changes to stem and progenitor cells, which are revealed only later on (Bracci et al., 2014; Johnston et al., 2013; Kusunoki and Hayashi, 2008; Li and Slayton, 2013). Despite this possibility, our studies are consistent with the idea that the developmental period constitutes a time during which the immune system is sensitive to modulation by environmental factors (Dietert, 2005; Dietert and Zelikoff, 2008; Luebke et al., 2006; Winans et al., 2011).

In summary, we report a study of developmental exposure to a mixture of chemicals associated with UOG operations on immune system development and function using three broad types of disease models: infection, allergic, and autoimmune. The major finding is that maternal exposure to this mixture durably affects the immune system of the offspring. Some of the observed changes were subtle, such as alterations in the number or percentage of certain cell types, whereas other changes were more manifest, such as advancement in the onset and severity of disease. Also, some alterations appeared to be more evident in the female offspring. Collectively, our findings suggest that developmental exposure to chemicals associated with UOG likely causes long-lasting changes in the mouse immune system.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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SUPPLEMENTAL MATERIALS

Developmental exposure to a mixture of 23 chemicals associated with unconventional oil and gas operations alters the immune system of C57Bl/6 mice

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	Control	0.1 µg/mL	p-value ^a	1 µg/mL	p-value ^a
Time to Parturition	19.0 ± 0.00	18.9 ± 0.18	0.89	19.0 ± 0.00	1.0
Dam Weight GD0	18.3 ± 0.24	18.6 ± 0.48	0.82	18.4 ± 0.32	0.96
Dam Weight GD7	20.8 ± 0.35	21.5 ± 0.47	0.39	21.3 ± 0.32	0.68
Dam Weight GD14	27.6 ± 0.35	29.2 ± 0.69	0.14	29.2 ± 0.42	0.18
Dam Weight PND2	25.1 ± 0.42	26.0 ± 0.48	0.28	26.3 ± 0.29	0.17

Supplemental Materials, Table S1. Mean time to parturition and dam body weight before, during, and 2 days after giving birth. Time to parturition indicates the number of days between gestational day (GD) 0 and birth of pups. Dams were weighed on GD0, 7, 14, and 2 days after parturition (PND2). Data represent the means from 10 dams in each treatment group. ^aCompared to dams drinking control water.

	Female 0.1µg/mL vs. Control			Male 0.1µg/mL vs. Control			Female 1 µg/mL vs. Control		Male 1 µg/mL vs. Control		Female vs. Male Control	Female vs. Male 0.1 µg/mL	Female vs. Male 1µg/mL
Cell type	Control	0.1µg/mL	p-value ^a	Control	0.1µg/mL	p-value ^a	1 µg/mL	p-value ^a	1 µg/mL	p-value ^a	p-value ^b	p-value ^b	p-value ^b
MLN Cells													
Cell Number	7708333 ± 1097424	8505000 ± 1137502	0.87	5128500 ± 518984	4698750 ± 430391	0.85	7533150 ± 1040419	0.99	5384500 ± 972929	0.95	0.36	0.01*	0.15
CD4 ⁺ T cells	1450263 ± 168394	128895 ± 179973	0.78	637990 ± 90913	534007 ± 38120	0.64	1281035 ± 150390	0.76	667462 ± 107956	0.97	0.001*	0.001*	0.005*
Th2 cells	22181 ± 3585	27799 ± 3679	0.49	7975 ± 1008	9084 ± 469	0.76	20802 ± 2920	0.96	8137 ± 1669	0.99	0.003*	<0.0001*	0.002*
Th17 cells	25316 ± 3239	56982 ± 9006	0.008*	12343 ± 4758	11248 ± 2316	0.71	37912 ± 7257	0.39	15922 ± 4838	0.81	0.001*	0.01*	0.52
Tregs	117598 ± 12010	88245 ± 12673	0.26	60761 ± 11006	46341 ± 5405	0.51	97642 ± 13330	0.53	62711 ± 10343	0.99	0.01*	0.01*	0.06
BAL cells													
Macrophages	44808 ± 8681	179845 ± 32636	0.002*	92538 ± 18232	121104 ± 25160	0.61	126768 ± 23430	0.047*	112601 ± 18138	0.79	0.69	0.17	0.63
Eosinophils	174123 ± 63817	476702 ± 93240	0.02*	421327 ± 102220	662437 ± 150330	0.32	305157 ± 45367	0.41	538508 ± 72394	0.77	0.52	0.32	0.01*
Neutrophils	18141 ± 7747	83090 ± 34974	0.12	31046 ± 9986	35931 ± 9277	0.94	49694 ± 10276	0.59	40424 ± 13096	0.82	0.99	0.19	0.58
Lymphocytes	40426 ± 13250	95771 ± 17938	0.04*	48126 ± 12505	71727 ± 24996	0.64	54381 ± 11835	0.78	56912 ± 12799	0.94	0.99	0.45	0.88

Supplemental Materials, Table S2. The number of immune cell populations in male and female offspring after HDM sensitization and challenge.

At maturity (6-8 weeks of age), 9-10 female and 9-10 male offspring from each developmental exposure group were sensitized and challenged with house dust mite (HDM). Within each group, offspring of the same sex were from a different treated dam. Flow cytometry was used to identify CD4⁺ T cells in the MLN, based on the following parameters: as CD4⁺ T cells (CD3⁺CD4⁺); Th2 cells (GATA3⁺CD4⁺); Th17 cells (RORγt⁺CD4⁺); Tregs (Foxp3⁺CD25⁺CD4⁺). Differential cell counting was used to enumerate eosinophils, macrophages, lymphocytes, and neutrophils in the BAL. The mean number of cells ± SEM is shown. ^aMixture-exposed group compared to control group within the indicated sex. ^bFemale versus male offspring of dams given the indicated concentration of the mixture. *Demarcates $p \leq 0.05$.

	Female 0.1 µg/mL vs. Control			Male 0.1 µg/mL vs. Control			Female 1 µg/mL vs. Control		Male 1 µg/mL vs. Control		Female vs. Male Control	Female vs. Male 0.1 µg/mL	Female vs. Male 1µg/mL
Cell type	Control	0.1µg/mL	p-value ^a	Control	0.1µg/mL	p-value ^a	1 µg/mL	p-value ^a	1 µg/mL	p-value ^a	p-value ^b	p-value ^b	p-value ^b
MLN Cells													
CD4 ⁺ T cells	19.3 ± 1.04	15 ± 0.56	0.02*	12 ± 0.91	12 ± 0.62	0.80	18 ± 1.2	0.39	13 ± 0.61	0.92	<0.0001*	0.048*	0.004*
Th2 cells	1.5 ± 0.09	2.2 ± 0.15	0.0003*	1.4 ± 0.21	1.8 ± 0.20	0.20	1.6 ± 0.09	0.82	1.2 ± 0.09	0.77	0.99	0.37	0.45
Th17 cells	2.01 ± 0.234	3.7 ± 0.35	0.001*	1.7 ± 0.32	2.4 ± 0.66	0.75	2.99 ± 0.277	0.04*	2.65 ± 1.04	0.61	0.40	1.0	0.98
Tregs	8.3 ± 0.29	6.8 ± 0.19	0.005*	9.3 ± 0.44	8.5 ± 0.73	0.46	7.6 ± 0.37	0.24	9.3 ± 0.22	1.0	0.50	0.08	0.06
Treg:Th2	5.8 ± 0.43	3.2 ± 0.21	<0.0001*	8.8 ± 1.5	5.4 ± 0.75	0.09	4.9 ± 0.35	0.003*	8.4 ± 0.88	0.98	0.13	0.42	0.049*
Treg:Th17	5.6 ± 0.51	2.9 ± 0.25	0.0001*	6.7 ± 0.80	6.05 ± 1.22	0.89	3.5 ± 0.35	0.001*	6.19 ± 1.02	0.93	0.01*	0.13	0.01*
BAL cells													
Macrophages	0.24 ± 0.05	0.24 ± 0.04	0.99	0.26 ± 0.07	0.17 ± 0.03	0.63	0.23 ± 0.02	0.98	0.23 ± 0.07	0.94	1.00	0.22	0.93
Eosinophils	0.51 ± 0.06	0.55 ± 0.04	0.78	0.62 ± 0.07	0.696 ± 0.15	0.67	0.58 ± 0.02	0.43	0.66 ± 0.07	0.92	0.72	0.03*	0.35
Neutrophils	0.06 ± 0.01	0.08 ± 0.02	0.40	0.04 ± 0.01	0.06 ± 0.01	0.81	0.09 ± 0.01	0.30	0.05 ± 0.02	0.99	0.99	0.42	0.01*
Lymphocytes	0.13 ± 0.02	0.12 ± 0.02	0.95	0.08 ± 0.01	0.07 ± 0.01	0.90	0.09 ± 0.01	0.27	0.07 ± 0.01	0.77	0.09	0.01*	0.10

Supplemental Materials, Table S3. The percentage of immune cell populations in male and female offspring after HDM sensitization and challenge. At maturity (6-8 weeks of age), 9-10 female and 9-10 male offspring from each developmental exposure group were sensitized and challenged with house dust mite (HDM). Within each group, offspring of the same sex were from a different treated dam. Flow cytometry was used to identify CD4⁺ T cells in the MLN, based on the following parameters: as CD4⁺ T cells (CD3⁺CD4⁺); Th2 cells (GATA3⁺CD4⁺); Th17 cells (RORγt⁺CD4⁺); Tregs (Foxp3⁺CD25⁺CD4⁺). Differential cell counting was used to enumerate eosinophils, macrophages, lymphocytes, and neutrophils in the BAL. The mean percentage of the indicated cell type ± SEM is shown. ^aMixture-exposed group compared to control group within the indicated sex. ^bFemale versus male offspring of dams given the indicated concentration of the mixture. *Demarcates p ≤ 0.05.

	Female 0.1µg/mL vs. Control			Male 0.1µg/mL vs. Control			Female 1µg/mL vs. Control		Male 1µg/mL vs. Control		Female vs. Male Control	Female vs. Male 0.1 µg/mL	Female vs. Male 1µg/mL
Cell type	Control	0.1µg/mL	P-value ^a	Control	0.1µg/mL	P-value ^a	1µg/mL	P-value ^a	1µg/mL	P-value ^a	p-value ^b	p-value ^b	p-value ^b
Cell Number	4897563 ± 944506	4830563 ± 1421214	0.99	5818714 ± 1289135	5909625 ± 687895	0.99	3858214 ± 1020211	0.81	4029750 ± 525796	0.33	0.99	0.51	0.69
CD4 ⁺ T cells	749418 ± 134060	736576 ± 226789	0.99	712501 ± 150731	850933 ± 79669	0.64	559500 ± 118212	0.72	539570 ± 85843	0.50	1.00	0.64	0.88
Th1 cells	43093.3 ± 12678	34762 ± 10766	0.84	61928.9 ± 15790	69719 ± 11045	0.87	25216.2 ± 5612	0.47	41727 ± 3981	0.41	0.82	0.04*	0.03*
Th2 cells	335.9 ± 101.9	430.9 ± 120.9	0.77	3267.1 ± 899	4587 ± 519	0.31	183.7 ± 54.8	0.55	2915 ± 384	0.92	0.001*	<0.0001*	<0.0001*
Th17 cells	12406.2 ± 3226.3	14386.1 ± 4379.7	0.91	4813 ± 982	5059.4 ± 786	0.98	8871 ± 1786	0.75	4667 ± 1215	0.99	0.31	0.05	0.12
Tregs	79722.7 ± 15778	72622.8 ± 20524	0.96	99543 ± 20971	103334 ± 12872	0.98	73906.2 ± 17487	0.97	79909 ± 11096	0.64	0.96	0.23	0.60
Tfh cells	2994.6 ± 923.7	5248.8 ± 3062	0.73	28858 ± 8971	31237 ± 7782	0.97	3739.8 ± 1654.2	0.97	17829 ± 2785	0.51	0.01*	0.01*	0.001*
CD8 ⁺ T cells	181834 ± 29673	216851 ± 74308	0.88	572043 ± 118587	666815 ± 61383	0.71	156679 ± 35859	0.94	431571 ± 68438	0.48	0.004*	0.0004*	0.002*
CTL	7429.8 ± 1693.8	8468.3 ± 3161.1	0.98	76117 ± 22812	57961 ± 11293	0.64	12901.9 ± 5512.5	0.55	34918 ± 4610	0.13	0.0004*	0.001*	0.02*
NP specific CD8 ⁺ T cells	3057 ± 543	3329 ± 757	0.98	11143 ± 3012	9303 ± 1713	0.79	4341 ± 1574	0.65	6553 ± 960	0.19	0.01*	0.01*	0.20

Supplemental Materials, Table S4. The number of T cell sub-populations in male and female offspring after infection. At maturity (6-8 weeks of age), 9-10 female and 9-10 male offspring from each developmental exposure group were infected (i.n.) with influenza A virus (HKx31; H3N2). Within each group, offspring of the same sex were from a different treated dam. Flow cytometry was used to identify T cells in the MLN, based on the following parameters: CD8⁺ T cells (CD3⁺CD8⁺); CTL (CD44^{hi}CD62L^{lo}CD8⁺); NP-specific CD8⁺ T cells (D^bNP₃₆₆₋₃₇₄⁺CD8⁺); CD4⁺ T cells (CD3⁺CD4⁺); Th2 cells (GATA3⁺CD4⁺); Th17 cells (RORγt⁺CD4⁺); Tfh (CXCR5⁺PD-1⁺ CD4⁺); Tregs (Foxp3⁺CD25⁺CD4⁺). The mean number ± SEM are shown. ^aMixture-exposed group compared to control group within the indicated sex. ^bFemale versus male offspring from dams given the indicated concentration of the mixture. *Demarcates p ≤ 0.05.

	Female 0.1 µg/mL vs. Control			Male 0.1 µg/mL vs. Control			Female 1 µg/mL vs. Control		Male 1 µg/mL vs. Control		Female vs. Male Control	Female vs. Male 0.1 µg/mL	Female vs. Male 1µg/mL
Cell type	Control	0.1µg/mL	p-value ^a	Control	0.1µg/mL	p-value ^a	1µg/mL	p-value ^a	1µg/mL	p-value ^a	p-value ^b	p-value ^b	p-value ^b
CD4 ⁺ T cells	15.5 ± 1.04	14.8 ± 1.18	0.89	12.8 ± 0.850	15 ± 1.03	0.18	16 ± 1.1	0.93	13.1 ± 0.538	0.97	0.43	1.0	0.30
Th1 cells	0.82 ± 0.11	0.67 ± 0.14	0.66	1.04 ± 0.101	1.1 ± 0.11	0.80	0.66 ± 0.11	0.65	1.12 ± 0.133	0.89	0.81	0.07	0.12
Th2 cells	0.006 ± 0.001	0.008 ± 0.002	0.58	0.056 ± 0.005	0.079 ± 0.006	0.004*	0.005 ± 0.001	0.89	0.073 ± 0.003	0.04 *	<0.0001*	<0.0001*	<0.0001*
Th17 cells	0.303 ± 0.111	0.343 ± 0.123	0.96	0.11 ± 0.03	0.09 ± 0.02	0.88	0.31 ± 0.08	0.99	0.11 ± 0.02	0.99	0.53	0.22	0.48
Tregs	1.6 ± 0.18	1.5 ± 0.14	0.91	1.8 ± 0.11	1.8 ± 0.13	0.99	2.1 ± 0.19	0.19	2.05 ± 0.234	0.58	0.98	0.90	1.0
Tfh cells	0.06 ± 0.02	0.07 ± 0.02	0.77	0.44 ± 0.08	0.48 ± 0.08	0.93	0.07 ± 0.02	0.89	0.45 ± 0.06	0.99	<0.0001*	<0.0001*	0.0001*
CD8 ⁺ T cells	3.9 ± 0.41	4.1 ± 0.45	0.90	10.4 ± 0.835	11.7 ± 0.635	0.40	4.4 ± 0.26	0.67	10.5 ± 0.498	0.99	<0.0001*	<0.0001*	<0.0001*
CTL	0.15 ± 0.01	0.15 ± 0.02	0.99	1.2 ± 0.25	0.94 ± 0.13	0.56	0.26 ± 0.07	0.10	0.995 ± 0.212	0.69	<0.0001*	0.003*	0.01*
NP specific CD8 ⁺ T cells	0.065 ± 0.004	0.08 ± 0.01	0.44	0.22 ± 0.04	0.16 ± 0.02	0.29	0.11 ± 0.01	0.005*	0.16 ± 0.01	0.35	<0.0001*	0.08	0.53

Supplemental Materials, Table S5. The percentage of T cell sub-populations in male and female offspring after infection. At maturity (6-8 weeks of age), 9-10 female and 9-10 male offspring from each developmental exposure group were infected (i.n.) with influenza A virus (HKx31; H3N2). Within each group, offspring of the same sex were from a different treated dam. Flow cytometry was used to identify the indicated T cell types in the MLN, based on the following parameters: CD8⁺ T cells (CD3⁺CD8⁺); CTL (CD44^{hi}CD62L^{lo}CD8⁺); NP-specific CD8⁺ T cells (D^bNP₃₆₆₋₃₇₄⁺CD8⁺); CD4⁺ T cells (CD3⁺CD4⁺); Th2 cells (GATA3⁺CD4⁺); Th17 cells (RORγt⁺CD4⁺); Tfh (CXCR5⁺PD-1⁺CD4⁺); Tregs (Foxp3⁺CD25⁺CD4⁺). The mean percentage ± SEM is shown. ^aMixture-exposed group compared to control group within the indicated sex. ^bFemale versus male offspring from dams given the indicated concentration of the mixture. *Demarcates p ≤ 0.05.

	Female 0.1µg/mL vs. Control			Male 0.1µg/mL vs. Control			Female 1µg/mL vs. Control		Male 1µg/mL vs. Control		Female vs. Male Control	Female vs. Male 0.1 µg/mL	Female vs. Male 1µg/mL
Cell type	Control	0.1µg/mL	p-value ^a	Control	0.1µg/mL	p-value ^a	p-value ^b	p-value ^a	1µg/mL	p-value ^a	p-value ^b	p-value ^b	p-value ^b
DAY 7													
MLN cells	9978571 ± 1246035	13037500 ± 1031675	0.21	11778571 ± 1486431	13103125 ± 1663959	0.74	13396875 ± 1605984	0.15	11818750 ± 1030622	0.99	0.95	0.92	0.41
CD4 ⁺ T cells	1525836 ± 219598	1658047 ± 188466	0.91	1803307 ± 183874	2005097 ± 305007	0.84	1970091 ± 257145	0.37	1748992 ± 222254	0.99	0.97	0.90	0.98
Th1 cells	90810 ± 30735	162232 ± 34164	0.22	50130 ± 13281	37254 ± 5272	0.58	94160 ± 19381	0.99	41334 ± 7628	0.77	0.79	0.01*	0.52
Th2 cells	1673 ± 337	1313 ± 185	0.81	1770 ± 224	2815 ± 974	0.48	1997 ± 566	0.84	1279.9 ± 224.2	0.19	1.00	0.27	0.91
Th17 cells	69154 ± 5892	72420 ± 15035	0.99	74398 ± 6687	80427 ± 8591	0.88	116230 ± 22938	0.15	78659 ± 10310	0.94	0.99	1.0	0.36
Tregs	130587 ± 16380	159616 ± 19831	0.68	160451 ± 15644	213183 ± 31461	0.28	200500 ± 31538	0.13	163910 ± 17532	0.99	0.96	0.83	0.88
DAY 21													
MLN cells	13103571 ± 1545669	12406594 ± 1380055	0.96	11545625 ± 2873425	10905625 ± 2157050	0.98	14875313 ± 2150489	0.76	14059375 ± 2080860	0.74	0.86	0.99	0.99
CD4 ⁺ T cells	2135186 ± 280250	1729275 ± 192064	0.58	1688515 ± 353411	1567659 ± 287275	0.96	2364883 ± 1615598	0.84	2050241 ± 283712	0.69	0.48	0.99	0.81
Th1 cells	67862 ± 16381	94961 ± 24133	0.57	19248 ± 4454	20392 ± 5197	0.99	65723 ± 12496	0.99	28353 ± 4974	0.40	0.05	0.95	0.89
Th2 cells	2177 ± 349	1234 ± 153	0.09	415 ± 115	375 ± 94	0.97	1882 ± 365	0.77	611 ± 144	0.49	0.0003*	0.52	0.13
Th17 cells	123129 ± 21883	91393 ± 11430	0.51	55322 ± 17380	53921 ± 13712	0.99	141889 ± 24496	0.79	85498 ± 15768	0.38	0.02*	0.60	0.21
Tregs	233309 ± 36518	193893 ± 25318	0.73	231943 ± 53240	217953 ± 42181	0.97	270879 ± 44271	0.75	283672 ± 40044	0.71	0.74	1.0	0.97
DAY 42													
MLN cells	16756250 ± 3019859	15902778 ± 2171435	0.96	9770000 ± 1710798	23322222 ± 3489954	0.002*	18863889 ± 1647874	0.80	15780500 ± 2086808	0.21	0.34	0.27	0.94
CD4 ⁺ T cells	3006056 ± 546306	2827342 ± 410654	0.95	1710725 ± 277970	3362214 ± 484674	0.01*	3462128 ± 279403	0.73	2379627 ± 282119	0.37	0.19	0.92	0.34
Th1 cells	112823 ± 28704	92899 ± 16695	0.77	71941 ± 14380	186874 ± 34465	0.02*	108376 ± 14302	0.99	117446 ± 30449	0.46	0.85	0.10	0.99
Th2 cells	1918 ± 388	1198 ± 186	0.25	890 ± 185	1595 ± 276	0.05	2118 ± 326	0.89	1020 ± 133	0.89	0.07	0.88	0.03*
Th17 cells	172501 ± 26431	174585 ± 30157	0.99	37338 ± 9751	81499 ± 10623	0.01*	183473 ± 19926	0.95	64574 ± 10246	0.15	0.0001*	0.01*	0.001*
Tregs	368823 ± 59889	365668 ± 47395	0.99	228291 ± 37780	442805 ± 60917	0.01*	457275 ± 36033	0.41	329128 ± 40734	0.28	0.32	0.86	0.38

Supplemental Materials, Table S6. The number of CD4⁺ T cell subsets in developmentally exposed mice after MOG peptide administration.

At maturity (6-8 weeks of age), EAE was induced in 8-10 female and 8-10 male offspring from each developmental exposure group. Within each group, offspring of the same sex were from a different treated dam. Mice were sacrificed 7, 21 or 42 days after MOG peptide administration, and flow cytometry was used to identify CD4⁺ T cells in the cervical lymph nodes, based on the following parameters: CD4⁺ T cells (CD3⁺CD4⁺); Th1 cells (Tbet⁺CD4⁺); Th2 cells (GATA3⁺CD4⁺); Th17 cells (RORγt⁺CD4⁺); Treg (Foxp3⁺CD25⁺CD4⁺). The mean percentage ± SEM is shown.

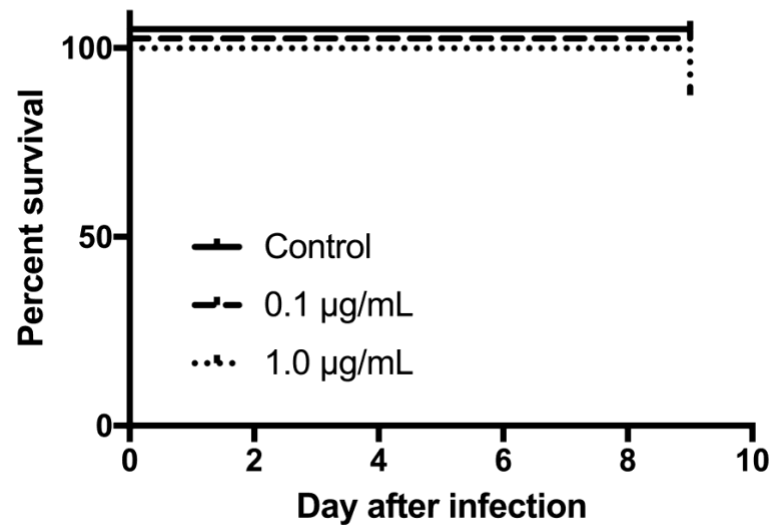
^aMixture-exposed group compared to control group within the indicated sex. ^bFemale versus male offspring from dams given the indicated concentration of the mixture. *Demarcates $p \leq 0.05$.

	Female 0.1µg/mL vs. Control			Male 0.1µg/mL vs. Control			Female 1µg/mL vs. Control		Male 1µg/mL vs. Control		Female vs. Male Control	Female vs. Male 0.1 µg/mL	Female vs. Male 1µg/mL
Cell type	Control	0.1µg/mL	P-value ^a	Control	0.1µg/mL	P-value ^a	1µg/mL	P-value ^a	1µg/mL	P-value ^a	p-value ^b	p-value ^b	p-value ^b
DAY 7													
CD4 ⁺ T cells	15 ± 0.67	13 ± 0.49	0.03*	16 ± 1.7	15 ± 0.69	0.87	15 ± 0.71	0.93	15 ± 1.2	0.76	0.98	0.57	1.0
Th1 cells	0.86 ± 0.25	1.3 ± 0.28	0.47	0.42 ± 0.09	0.29 ± 0.03	0.24	0.698 ± 0.142	0.87	0.33 ± 0.03	0.52	0.33	0.01*	0.68
Th2 cells	0.017 ± 0.002	0.010 ± 0.001	0.08	0.016 ± 0.002	0.018 ± 0.004	0.82	0.015 ± 0.003	0.77	0.011 ± 0.001	0.49	0.99	0.23	0.84
Th17 cells	0.75 ± 0.06	0.54 ± 0.09	0.21	0.67 ± 0.07	0.63 ± 0.04	0.89	0.86 ± 0.09	0.61	0.67 ± 0.06	0.99	0.99	0.99	0.47
Tregs	1.3 ± 0.04	1.2 ± 0.09	0.77	1.5 ± 0.17	1.6 ± 0.07	0.68	1.5 ± 0.11	0.34	1.4 ± 1.1	0.97	0.95	0.65	0.99
Treg:Th1	2.9 ± 0.78	1.2 ± 0.18	0.15	4.3 ± 0.79	6.1 ± 0.84	0.20	2.9 ± 0.67	0.99	4.5 ± 0.53	0.97	0.65	0.01*	0.72
Treg:Th2	84 ± 10	126 ± 12	0.34	95 ± 9.2	116 ± 19	0.66	133 ± 33	0.24	143 ± 18	0.13	0.99	0.97	0.99
Treg:Th17	1.8 ± 0.14	2.6 ± 0.32	0.05	2.2 ± 0.19	2.6 ± 0.19	0.41	1.8 ± 0.15	0.99	2.2 ± 0.29	0.98	0.94	0.99	0.70
DAY 21													
CD4 ⁺ T cells	16 ± 0.57	14 ± 0.65	0.15	16 ± 1.2	15 ± 0.69	0.56	16 ± 0.62	0.99	15 ± 0.71	0.62	0.94	0.82	0.26
Th1 cells	4.1 ± 1.1	5.8 ± 1.7	0.57	1.1 ± 0.14	1.2 ± 0.13	0.96	3.2 ± 0.73	0.88	1.5 ± 0.39	0.50	0.50	0.69	0.98
Th2 cells	0.11 ± 0.01	0.07 ± 0.01	0.04*	0.027 ± 0.005	0.022 ± 0.003	0.68	0.08 ± 0.01	0.24	0.029 ± 0.004	0.87	<0.0001*	0.03*	0.04*
Th17 cells	5.7 ± 0.38	5.3 ± 0.46	0.79	2.9 ± 0.41	3.4 ± 0.38	0.61	6.04 ± 0.378	0.82	4.1 ± 0.32	0.09	<0.0001*	<0.0001*	0.0001*
Tregs	10.5 ± 0.608	11 ± 0.56	0.76	13 ± 0.47	14 ± 0.39	0.67	11 ± 0.47	0.54	14 ± 0.64	0.75	0.84	0.99	0.93
Treg:Th1	5.2 ± 0.85	5.23 ± 1.2	1.0	13 ± 1.3	13 ± 1.5	0.98	6.04 ± 0.954	0.84	11 ± 1.4	0.73	0.07	0.41	0.78
Treg:Th2	122 ± 23	168 ± 16	0.40	739 ± 232	720 ± 102	0.99	194 ± 32	0.11	541 ± 75	0.64	0.01*	0.03*	0.47
Treg:Th17	1.9 ± 0.10	2.3 ± 0.73	0.13	5.2 ± 0.63	4.5 ± 0.51	0.59	1.96 ± 0.113	0.95	3.5 ± 0.28	0.07	<0.0001*	0.0004*	0.10
DAY 42													
CD4 ⁺ T cells	18 ± 0.57	17.8 ± 0.951	0.92	18 ± 1.3	15 ± 0.95	0.06	18 ± 0.32	0.95	16 ± 0.77	0.18	1.00	0.20	0.22
Th1 cells	3.4 ± 0.34	3.2 ± 0.32	0.83	4.2 ± 0.61	5.5 ± 0.54	0.41	3.1 ± 0.22	0.71	4.7 ± 0.87	0.87	0.92	0.35	0.82
Th2 cells	0.06 ± 0.01	0.04 ± 0.01	0.06	0.01 ± 0.01	0.047 ± 0.003	0.69	0.06 ± 0.01	0.79	0.044 ± 0.003	0.44	0.92	0.99	0.08
Th17 cells	6.01 ± 0.384	6.03 ± 0.364	0.99	2.03 ± 0.260	2.5 ± 0.28	0.47	5.3 ± 0.43	0.44	2.7 ± 0.32	0.28	<0.0001*	<0.0001*	<0.0001*
Tregs	13 ± 0.71	13 ± 0.37	0.79	13 ± 0.26	13 ± 0.34	0.99	13 ± 0.34	0.78	14 ± 0.43	0.64	0.95	0.34	0.51
Treg:Th1	4.01 ± 0.445	4.5 ± 0.46	0.69	3.8 ± 0.56	2.7 ± 0.35	0.24	4.4 ± 0.31	0.74	3.6 ± 0.45	0.94	0.99	0.07	0.74
Treg:Th2	217 ± 25	343 ± 49	0.09	324 ± 60	298 ± 25	0.89	256 ± 38	0.77	330 ± 24	0.89	0.44	0.97	0.78
Treg:Th17	2.1 ± 0.07	2.3 ± 0.21	0.82	7.8 ± 1.1	5.7 ± 0.47	0.22	2.6 ± 0.19	0.16	5.9 ± 0.73	0.27	<0.0001*	0.01*	0.01*

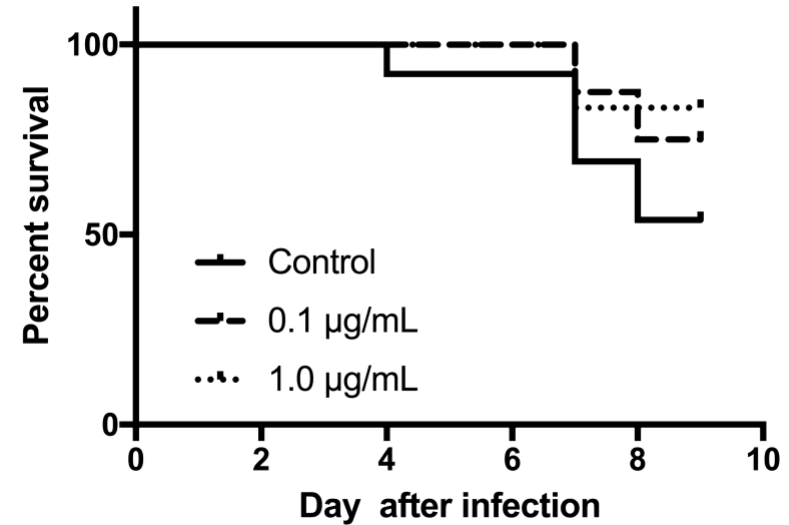
Supplemental Materials, Table S7. The percentage of CD4⁺ T cell subsets in developmentally exposed mice after MOG peptide administration.

At maturity (6-8 weeks of age), EAE was induced in 8-10 female and 8-10 male offspring from each developmental exposure group. Within each group, offspring of the same sex were from a different treated dam. Mice were sacrificed 7, 21 or 42 days after MOG peptide administration, and flow cytometry was used to identify CD4⁺ T cells in the cervical lymph nodes, based on the following parameters: CD4⁺ T cells (CD3⁺CD4⁺); Th1 cells (Tbet⁺CD4⁺); Th2 cells (GATA3⁺CD4⁺); Th17 cells (RORγt⁺CD4⁺); Treg (Foxp3⁺CD25⁺CD4⁺). The mean percentage ± SEM is shown. ^aMixture-exposed group compared to control group within the indicated sex. ^bFemale versus male offspring from dams given the indicated concentration of the mixture. *Demarcates p ≤ 0.05.

A. Female offspring



B. Male offspring



Supplemental Materials, Figure S1. Effects of developmental exposure to chemicals associated with UOG on survival following primary influenza virus infection. At maturity (6-8 weeks of age), 9-10 female and 9-10 male offspring from each exposure group infected with influenza A virus (HKx31; H3N2). Within each group, offspring of the same sex were from a different treated dam. Post infection survival for (A) female and (B) male offspring was recorded daily for 9 days. There were no statistically significant differences in survival among same sex offspring.