

## Animals

Female B<sub>6</sub>C<sub>3</sub>F<sub>1</sub> mice were used in these studies as they are the National Toxicology Program preferred strain for evaluating general toxicity. All mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 7-8 weeks of age. Upon arrival, the animals were allowed to acclimate for a minimum of 5 days. All animals were randomly assigned to treatment groups, weighed, and individually identified via tail marking using a permanent marker. Dose groups were identified by cage cards. Both the dosing group as well as the animal numbers were identified on each cage. The animals were housed 5 mice/cage in ventilated plastic shoe box cages with hardwood chip bedding, modified NIH-31 6% irradiated rodent diet (Harlan Teklad – item #7913) and sterile tap water from water bottles *ad libitum*. The temperature in the animal facility was maintained between 65 and 78 °F and the relative humidity between 30 and 70%; a light/dark cycle was maintained at 12-hr intervals. All animal experiments were performed in an AAALAC International accredited National Institute for Occupational Safety and Health (NIOSH) animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

## Test articles and chemicals

Acetone [CAS #67-64-1] was purchased from Sigma-Aldrich. Perfluorohexane sulfonic acid (95%, PFHxS) [CAS #355-46-4] was purchased from Synquest Laboratories. PFHxS concentrations were selected based on an initial 7-day range finding study and previous studies conducted with the short-chain PFAS PFPeA, PFHxA, and PFHpA (Weatherly et al. 2023) and the long-chain PFAS PFOA (Shane et al. 2020).

## PFAS exposures

For study A, B<sub>6</sub>C<sub>3</sub>F<sub>1</sub> mice (5/group) were topically treated on the dorsal surface of each ear (25 µl/ear) with vehicle (acetone) or PFHxS concentrations (1.25, 2.5, 5% w/v) once a day for 28 consecutive days. These studies were conducted for the purpose of hazard identification and the concentrations of PFHxS were based on concentration range finding studies that showed effects in the absence of overt toxicity. Body weights were measured weekly before exposure to ensure no overt toxicity was occurring due to PFAS exposure. Animals were euthanized by CO<sub>2</sub> asphyxiation approximately 24 hrs after the last exposure.

For study B, B<sub>6</sub>C<sub>3</sub>F<sub>1</sub> mice (5/group) were topically treated on the dorsal surface of each ear (25 µl/ear) with vehicle (acetone) or PFHxS concentrations (0.625, 1.25, 2.5% w/v) once a day for 10 consecutive days. A decrease in PFHxS concentration and time of exposure was used in these studies as no trends in weight loss was observed by day 10 with these concentrations. Four days prior to euthanasia, the mice were immunized with 7.5×10<sup>7</sup> SRBC (in 1 ml volume) by i.v. injection. All SRBC for these studies were drawn from a single donor animal (Lampire Laboratories; Pipersville, PA). Animals were euthanized by CO<sub>2</sub> asphyxiation approximately 24 hrs after the last exposure.

## **Tissue processing**

Following euthanasia, animals were weighed, and examined for gross pathology. The liver, spleen, kidneys, and thymus were removed, cleaned of connective tissue, and weighed. Left and right auricular draining lymph nodes (dLNs; draining the site of chemical application), spleen (1/2), and one ear pinna were collected and placed in 4 mL RPMI (Corning). Spleen (1/2), dLN (2 nodes/animal), and ear (1) pinna single cell suspensions for immune phenotyping were prepared as previously described (Weatherly et al. 2021). Half of one ear pinna was placed in 0.5 ml of RNAlater for subsequent gene expression analysis (see below). A small lobe of the liver (caudate) was collected in 0.5 ml of RNAlater for subsequent gene expression (see below). The remainder of the liver, spleen (1/2), ear pinna (1/2), and one kidney (right) were collected in 10% formalin for histopathology analysis.

## **Serum chemistries**

Blood samples were collected via cardiac puncture, transferred to serum separation tubes, and separated by centrifugation. The serum was frozen at -20 °C for subsequent serum chemistry analysis. Selected serum chemistries were evaluated using a Catalyst DX Chemistry Analyzer (IDEXX Laboratories, Inc.; Westbrook, ME). Endpoints analyzed included: alkaline phosphates (ALKP), urea nitrogen (BUN), glucose (GLU), and cholesterol (CHOL).

## **Analytical PFAS detection**

Serum and urine samples were collected and analyzed for individual PFHxS by Vista Analytical Laboratory, following Vista's standard operating procedures (Vista Analytical Laboratory, El Dorado Hills, CA) of solid phase extraction and liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described in (Shoemaker et al. 2008). Serum was collected from each animal and urine was pooled following euthanasia for each group of mice. The results for PFHxS include the linear isomer only. The Initial Calibration and Continuing Calibration Verifications met the acceptance criteria as described in (Shoemaker et al. 2008). No analytes were detected in the Method Blank above Reporting Limit. The labeled standard recoveries for all quality controls and samples were within the acceptance criteria as described in (Shoemaker et al. 2008).

## **Flow Cytometry**

For staining, single cell suspensions were resuspended in staining buffer containing  $\alpha$ -mouse CD16/32 antibody (Fc Block) (BD Biosciences) then incubated with a cocktail of fluorochrome-conjugated antibodies specific for mouse cell surface antigens. For dLN and spleen cells: B220-V500 (RA3-6B2), CD11b-PerCP-Cy5.5 (M1/70), CD8-PE-CF594 (53-6.7), Siglec-F-PE (E50-2440), Ly6G-FITC (1A8) (BD Biosciences), CD11c-eFluor 450 (N418), CD86-APC (GL1) (eBioscience), CD45-Superbright 780 (30-F11), F4/80-PE-Cy7 (BM8), MHCII-APC-eF780

(M5/114.15.2) (Invitrogen), CD4-BV711 (RM4-5), Ly6C-AF700 (HK1.4) (BioLegend). For ear cells: CD4-BV711 (RM4-5), NKp46-BV605 (29A1.4) (BioLegend), CD8-PE-CF594 (53-6.7), CD3-V500 (500A2), Ly6G-FITC (1A8), CD11b-PerCP-Cy5.5 (M1/70), CD11c-AF700 (HL3), Siglec-F-PE (E50-2440) (BD Biosciences), F4/80-PE-Cy7 (BM8), MHCII-APC-eF780 (M5/114.15.2), CD45-Superbright 780 (30-F11), FcεRI-APC (MAR-1) (Invitrogen), CD117-eFluor 450 (2B8) (eBioscience). Cells were then washed, fixed in Cytotfix buffer (BD Biosciences), resuspended in staining buffer, and a minimum of 100,000 CD45+ events were collected on a LSR II flow cytometer (BD Biosciences), compensation controls were prepared with eBioscience UltraComp eBeads. Analysis was performed using FlowJo v10 software (TreeStar Inc., Ashland, OR). All events were gated on single cells using FSC and SSC parameters prior to subsequent gating. Cellular populations were define using the gating strategies outlined below; Fluorescence minus ones (FMOs) were used as gating controls.

### Gating strategies for immunophenotyping

<b>Spleen and dLN cells</b>	
<b>Cell Type</b>	<b>Gating Strategy</b>
CD4 T cells	CD45 <sup>+</sup> , lymphocyte gate (FSC/SSC <sup>low</sup> ), CD8 <sup>-</sup> , B220 <sup>-</sup> , NKp46 <sup>-</sup> , CD4 <sup>+</sup>
CD8 T cells	CD45 <sup>+</sup> , lymphocyte gate (FSC/SSC <sup>low</sup> ), CD4 <sup>-</sup> , B220 <sup>-</sup> , NKp46 <sup>-</sup> , CD8 <sup>+</sup>
B cells	CD45 <sup>+</sup> , lymphocyte gate (FSC/SSC <sup>low</sup> ), CD4 <sup>-</sup> , CD8 <sup>-</sup> , NKp46 <sup>-</sup> , B220 <sup>+</sup> (MHCII/CD86 MFI increases with activation)
NK cells	CD45 <sup>+</sup> , lymphocyte gate (FSC/SSC <sup>low</sup> ), CD4 <sup>-</sup> , CD8 <sup>-</sup> , B220 <sup>-</sup> , NKp46 <sup>+</sup>
Eosinophils	CD45 <sup>+</sup> , SSC <sup>high</sup> , CD11b <sup>+</sup> , Ly6G <sup>low</sup> , Siglec-F <sup>+</sup>
Neutrophils	CD45 <sup>+</sup> , SSC <sup>high</sup> , CD11b <sup>+</sup> , Siglec-F <sup>-</sup> , Ly6G <sup>high</sup>
DCs	CD45 <sup>+</sup> , SSC <sup>int</sup> , MHCII <sup>+</sup> , CD11c <sup>+</sup> , F4/80 <sup>-</sup> (MHCII/CD86 MFI increases with activation)
CD11b <sup>+</sup> monocytes/macrophages	CD45 <sup>+</sup> , SSC <sup>int</sup> , CD11b <sup>+</sup> , SiglecF <sup>-</sup> , Ly6G <sup>-</sup> , Lyc6 <sup>+/-</sup> , F4/80 <sup>+/-</sup>
<b>Skin (ear) cells</b>	
<b>Cell Type</b>	<b>Gating Strategy</b>
CD4 T cells	CD45 <sup>+</sup> , Lymphocyte gate (FSC/SSC <sup>low</sup> ), CD3 <sup>+</sup> , CD4 <sup>+</sup> , CD8 <sup>-</sup>
CD8 T cells	CD45 <sup>+</sup> , Lymphocyte gate (FSC/SSC <sup>low</sup> ), CD3 <sup>+</sup> , CD4 <sup>-</sup> , CD8 <sup>+</sup>
NK cells	CD45 <sup>+</sup> , Lymphocyte gate (FSC/SSC <sup>low</sup> ), CD3 <sup>-</sup> , NKp46 <sup>+</sup>
Eosinophils	CD45 <sup>+</sup> , SSC <sup>high</sup> , CD11b <sup>+</sup> , Ly6G <sup>low</sup> , Siglec-F <sup>+</sup>
Neutrophils	CD45 <sup>+</sup> , SSC <sup>high</sup> , CD11b <sup>+</sup> , Siglec-F <sup>-</sup> , Ly6G <sup>high</sup>
DCs	CD45 <sup>+</sup> , SSC <sup>int</sup> , MHCII <sup>+</sup> , CD11c <sup>+</sup> , F4/80 <sup>-</sup> , CD11b <sup>+/-</sup>

### Gene expression

Ear (half an ear pinna/mouse) and liver (caudate) were homogenized on a TissueLyser II in Buffer RLT (Qiagen). Total RNA was isolated using Qiagen's RNeasy mini spin column kits with DNase treatment on a QIAcube automated RNA isolation machine. RNA concentrations and purity were analyzed on a NanoDrop spectrophotometer (Thermo Fisher Scientific). The

cDNA (1-2 µg) was prepared on an Eppendorf Mastercycler using Applied Biosystems' High Capacity Reverse Transcription kit. The cDNA was used on the real-time RT<sup>2</sup> Profiler PCR Array Mouse Immunotoxicity Targets (QIAGEN; Product #: PAMM-179ZC) and Innate and Adaptive Immune Response (QIAGEN; Product #: PAMM-052ZC) (spleen) and peroxisome proliferator-activated receptor (PPAR) Targets and Hepatotoxicity (QIAGEN; Product #: PAMM-149ZC and PAMM-093ZC) (liver) and also used as template for real-time PCR reactions containing TaqMan PCR Master Mix with gene-specific primers (Applied Biosystems) on a 7500 Real-Time PCR System. Transcripts were chosen to be confirmed via TaqMan qPCR based on fold-change and number of concentrations that were altered. The cDNA was used as a template for real-time PCR reactions containing TaqMan PCR Master Mix with gene-specific primers (Applied Biosystems) on a 7500 Real-Time PCR System. Relative fold gene expression changes ( $2^{-\Delta\Delta CT}$ ) were determined compared to vehicle controls and normalized for expression of reference gene  $\beta$ -actin (Taqman).

## **Histology**

Each tissue sample stored in 10% formalin was embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin (H&E) and evaluated by a veterinary pathologist at StageBio (Mason, Ohio) using The Society of Toxicologic Pathology Guideline (Crissman et al. 2004). Provantis™ pathology software v10.2.3.1 was utilized for data capture and table generation. Histopathology grades were assigned as grade 1 (minimal), grade 2 (mild), grade 3 (moderate), grade 4 (marked), or grade 5 (severe) based on an increasing extent of change. Criteria used for skin grading was previously described (Weatherly et al. 2021).

## **Spleen IgM response to SRBC**

The primary IgM response to SRBC was enumerated using a modified hemolytic plaque assay (Jerne and Nordin 1963; Shane et al. 2020). Four days prior to euthanasia, the mice were immunized with  $7.5 \times 10^7$  SRBC (in 200 µl volume) by i.v. injection. On the day of sacrifice, mice were euthanized by CO<sub>2</sub> asphyxiation, body and organ weights were recorded, and spleens were collected in 3 mL of Hank's balanced salt solution (HBSS). Single cell suspensions of the spleens from individual animals were prepared in HBSS by disrupting the spleen between the frosted ends of microscopic slides. To identify the total number of spleen cells, 20 µl of cells were added to 10 mL of Isoton II diluent (1:500; Beckman Coulter, Brea, CA) and two drops of Zap-o-globin (Beckman Coulter, Brea, CA) were added to lyse red blood cells. Cells were then counted in the Coulter counter. Dilutions (1:30 and 1:120) of spleen cells were then prepared and 100 µl of each dilution were added to test tubes containing a 0.5 mL warm agar/dextran mixture (0.5% Bacto-Agar, DIFCO; and 0.05% DEAE dextran; Sigma, St. Louis, MO), 25 µl of 1:1 ratio of SRBC suspension, and 25 µl of 1:4 dilution (1 mL lyophilized) guinea pig complement (Cedarlane Labs, Burlington, Canada). Each sample was vortexed, poured into a petri dish, covered with a microscope coverslip, and incubated for 3 h at 37 °C. The plaques (representing antibody-forming B-cells) were then counted. Results were expressed in terms of both specific activity (IgM PFC per 10<sup>6</sup> spleen cells) and total activity (IgM PFC per spleen).

## Serum IgM response to SRBC

Serum samples were analyzed for anti-SRBC IgM using a commercially available ELISA kit (Life Diagnostics, West Chester, PA), according to manufacturer recommendations with modifications. In brief, test serum was diluted (1:200, 1:400, 1:800 and 1:1,600) and incubated in the anti-SRBC coated microtiter wells for 45 min at 25 °C. The wells were subsequently washed, 100 µl horseradish peroxidase-conjugated secondary antibody was added, and the plates incubated for an additional 45 min at 25 °C. Thereafter, the wells were washed to remove unbound antibodies and 100 µl tetramethylbenzidine peroxidase (TMB) reagent was added to each well. The plates were incubated for 20 min at room temperature before color development was stopped by addition of 50 µl of kit-provided Stop Solution. Optical density in each well was then measured spectrophotometrically at 450 nm using a Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA). The concentration of anti-SRBC IgM in the test samples was determined by comparison to a standard curve generated in parallel using SoftMax Pro software and reported as units of anti-SRBC IgM (U/ml) plotted vs absorbance values at 450 nm.

- Crissman JW, Goodman DG, Hildebrandt PK, Maronpot RR, Prater DA, Riley JH, Seaman WJ, Thake DC. 2004. Best practices guideline: Toxicologic histopathology. *Toxicologic pathology*. 32(1):126-131.
- Jerne NK, Nordin AA. 1963. Plaque formation in agar by single antibody-producing cells. *Science (New York, NY)*. 140(3565):405.
- Shane HL, Baur R, Lukomska E, Weatherly L, Anderson SE. 2020. Immunotoxicity and allergenic potential induced by topical application of perfluorooctanoic acid (pfoa) in a murine model. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 136:111114.
- Shoemaker JA, Grimmer P, Boutin B. 2008. Determination of selected perfluorinated alkyl acids in drinking water by solid phase extraction and liquid chromatography/tandem mass spectrometry (lc/ms/ms). In: Agency USEP, editor. Washington, DC.
- Weatherly LM, Shane HL, Lukomska E, Baur R, Anderson SE. 2021. Systemic toxicity induced by topical application of heptafluorobutyric acid (pfba) in a murine model. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 156:112528.
- Weatherly LM, Shane HL, Lukomska E, Baur R, Anderson SE. 2023. Systemic toxicity induced by topical application of perfluoroheptanoic acid (pfhpa), perfluorohexanoic acid (pfhxa), and perfluoropentanoic acid (pfpea) in a murine model. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 171:113515.