

Materials and Methods

Animals

Female B₆C₃F₁ mice were used in these studies as they are the National Toxicology Program preferred strain for evaluating general toxicity. All mice were purchased from Taconic (Germantown, NY) 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. A preliminary analysis of variance on body weights was performed to insure homogenous distribution of animals across treatment groups. 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 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 Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) 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] and heptafluorobutyric acid (98%; PFBA) [CAS# 375-22-4] were purchased from Sigma-Aldrich. PFBA concentrations were selected based on an initial 7-day range finding study and the highest concentration that did not induce overt toxicity (plus two serial dilutions) were used for these studies.

PFBA exposures

For all studies, B₆C₃F₁ mice (5/group) were topically treated on the dorsal surface of each ear (25µl/ear) with acetone vehicle or concentrations of PFBA ranging from 3.75-7.5% w/v, once a day for 28 consecutive days. Due to skin sensitivity at the dosing site, the PFBA 15% group was dosed intermittently during the study on days 1-8, 14, 15, 17, 18, 22, 25, and 28 for a total of 15 days. Body weights were taken each day before exposure to ensure no overt toxicity was occurring due to PFBA exposure. Animals were euthanized by CO₂ asphyxiation 24 hrs after the last exposure.

Tissue processing

Animals were euthanized by CO₂ inhalation, 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) and spleen (1/2) were collected in 4 mL RPMI.

Spleen (1/2) and dLN (2 nodes/animal) cell suspensions were prepared by mechanical disruption of tissues between frosted microscope slides in RPMI and cells were counted after RBC lysis using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter). One ear pinna was collected and placed in 4 ml of RPMI for immune phenotyping and half of one ear was collected in 0.5ml of RNeasy lysis buffer for subsequent gene expression analysis (see below). Ear cell suspensions were prepared by splitting ear pinna into ventral and dorsal halves, followed by an enzymatic digestion for 90 min at 37 °C with 0.25 mg/ml Liberase-TL Research grade (Roche) in RPMI with 100 µg/ml DNase I (Sigma-Aldrich). Digestion was stopped by the addition of 3ml of RPMI + 10% fetal bovine sera (FBS), the ear pinna + media were transferred to gentleMACS C Tubes (Miltenyi Biotec), then cells were mechanically disrupted on a gentleMACS™ Dissociator (Miltenyi Biotec). Following disruption, cells were passed through a 70 µm cell strainer to make a single cell suspension, washed with RPMI 10% FBS, then live cells were counted on a Cellometer using AO/PI (Nexcelom) in order to quantify cells. A small lobe of the liver was collected in 0.5 ml of RNeasy lysis buffer for subsequent gene expression (see below). The remainder of the liver, spleen (1/2), ear (1/2), and one kidney was collected in 10% formalin for histopathology analysis.

Serum chemistries

Blood samples were collected via cardiac puncture, collected in serum collection 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: albumin (ALB), globulin (GLOB), alkaline phosphates (ALKP), alanine aminotransferase (ALT), urea nitrogen (BUN), glucose (GLU), total protein (TP), and cholesterol (CHOL).

Flow Cytometry

For staining, single cell suspensions were resuspended in staining buffer containing α-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 defined using the gating strategies outlined below; Fluorescence minus ones (FMOs) were used as gating controls.

Gating strategies for immunophenotyping

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

Gene expression

Ear (half ear/mouse) and liver (small lobe) 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 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. Relative fold gene expression changes ($2^{-\Delta\Delta CT}$) were determined compared to acetone controls and normalized for expression of housekeeping gene *beta-actin*.

Histology

Tissues were collected in 10% formalin for histopathology. Each tissue sample was embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin (H&E) and evaluated by a veterinary pathologist. 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. Liver histopathology was a subjective analysis, criteria used for skin grading is shown in below.

Histopathology grading criteria

<u>Dosing Site Scoring</u>	
Epidermal hyperplasia	
0- Normal	1-2 layers of keratinocytes
1- Minimal	3-4 layers of keratinocytes
2- Mild	5-6 layers of keratinocytes
3- Moderate	>6 layers of keratinocytes
Dermal Fibrosis	
0- Normal	
1 – Minimal	Increased thickness, but <2X control
2- Mild	2-3X control thickness
3 – Moderate	>3X control thickness
Epidermal Hyperkeratosis	
0- Absent	
1- Minimal –	<2X control of compacted keratin
2- Mild -	approx. 2X control amounts of compacted keratin
3- Moderate -	>2X control amounts of compacted keratin
Erosion/ulcer (associated with inflammation only)	
0- Absent	
1- Minimal –	One focus
2- Mild -	Two foci.
Dermal Inflammation	
0- Normal -	No increase in leukocytes compared to controls
1- Minimal -	Diffuse minimal increase in leukocyte density scattered in dermis or small focus associated with erosion/ulcer
2- Mild -	Above, with small accumulations of inflammatory cells in the dermis
Epidermal necrosis (coagulative)	
0- Absent	
1- Minimal -	Superficial loss of differential staining affecting <10% of dosed surface of ear.
2- Mild -	Loss of differential staining affecting <10% of dosed side of ear, full thickness in at least one area or superficial loss of differential staining for >10% of surface area.
3- Moderate -	Loss of differential staining affecting ~10-40% of surface area with full thickness in at least one area.
4- Marked -	Loss of differential staining affecting ~40-90% of surface area with full thickness in at least one area.