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Systemic toxicity induced by topical application of perfluoroheptanoic acid (PFHpA), perfluorohexanoic acid (PFHxA), and perfluoropentanoic acid (PFPeA) in a murine model

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic structurally diverse chemicals incorporated into industrial and consumer products. PFHpA, PFHxA, and PFPeA are carboxylic PFAS (C7, C6, C5, respectively) labeled as a safer alternative to legacy carboxylic PFAS due to their shorter half-life in animals. Although there is a high potential for dermal exposure, these studies are lacking. The present study conducted analyses of serum chemistries, immune phenotyping, gene expression, and histology to evaluate the systemic toxicity of a sub-chronic 28-day dermal exposure of alternative PFAS (1.25–5% or 31.25–125 mg/kg/dose) in a murine model. Liver weight (% body) significantly increased with PFHpA, PFHxA, and PFPeA exposure and histopathological changes were observed in both the liver and skin. Gene expression changes were observed with PPAR isoforms in the liver and skin along with changes in genes involved in steatosis, fatty acid metabolism, necrosis, and inflammation. These findings, along with significant detection levels in serum and urine, support PFAS-induced liver damage and PPARα, δ, and γ involvement in alternative PFAS systemic toxicity and immunological disruption. This demonstrates that these compounds can be absorbed through the skin and brings into question whether these PFAS are a suitable alternative to legacy PFAS.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of structurally diverse chemicals used in industrial and consumer products. Due to their amphiphilic properties, PFAS are used in flame-retardant products, water and grease repellent products, nonstick cookware, carpets, leather products, and firefighting foams (Lindstrom et al., 2011). The fluorinated hydrophobic carbon chain and hydrophilic head structure along with the carbon-fluoride bond strength also makes these compounds very stable and resistant to degradation (Kirsch, 2013). The above properties have led to detection and persistence in the environment and in the human body (Calafat et al., 2007; Lau et al., 2007). The legacy PFAS perfluorooctanoic acid (PFOA) and perfluorooctane sulfate (PFOS) are the two most common PFAS found in the environment and therefore the most studied. However, due to health concerns, efforts are in place to phase-out legacy PFAS by regulatory actions to eliminate their production (U.S. EPA 2022b).

Due to the phasing out of the legacy PFAS, alternatives are being used as replacement chemicals. Carboxylic PFAS are considered long-alkyl chain if they contain 7 or more carbons in their carbon chain. In humans, the long-chain PFAS (PFOA and PFOS, both containing 8 carbons) have half-lives of 2.1–10.1 and 3.3–27 years, respectively (ATSDR, 2021). Short-alkyl chain PFAS contain 6 or less carbons and can result from break-down products of long-chain PFAS (Li et al., 2020). Some of the most common alternative carboxylic PFAS include PFHpA, PFHxA, and PFPeA. PFHpA is an alternative PFAS containing 7 carbons with an estimated half-life of 62–70 days in humans (Russell et al., 2015; Xu et al., 2020) and PFHxA contains 6 carbons with an estimated half-life of 32 days in humans (Russell et al., 2013). PFPeA contains 5 carbons and although elevated levels in drinking water have been observed, serum levels in human populations have been below the limits of detection and no half-life has been established (Xu et al., 2020). These short-alkyl chain PFAS tend to be considered a safer alternative mainly due to their shorter half-life (Wang et al., 2013). It is important to note that PFOA, while having an 8-carbon chain, has 7 fully fluorinated carbons. Likewise, PFHpA, PFHxA, and PFPeA have 6, 5, and 4 fully fluorinated carbons, respectively.

While *in vivo* studies investigating alternative PFAS are scarce, select

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PFAS have been detected in human samples in epidemiology and occupational exposure studies (Göckener et al., 2020; Pérez et al., 2013). In fact, in human liver samples from Spain both PFHpA and PFHxA were found at higher concentrations than PFOA, and PFHxA occurred at the highest concentration in the liver and brain tissue out of the 20 PFAS analyzed (Pérez et al., 2013). Similar to toxicities associated with legacy PFAS, PFHxA has also been found to increase liver weight and decrease serum cholesterol levels in rats via oral exposure (Kirkpatrick, 2005). Both PFHxA and PFHpA were detected in human breast milk samples from women in the United States and the PFHxA concentrations were similar to that of PFOA in these samples (Zheng et al., 2021). Collectively, these limited studies indicate alternative PFAS human exposure is occurring and has the potential for toxicity.

While ingestion has been reported as the most common route of exposure to PFAS, there is potential for dermal exposure to alternative PFAS via commercial products, the environment, and occupation. PFHpA, PFHxA, and PFPeA have been detected in several consumer products through which dermal exposure could occur. These include carpets, gloves, nanosprays, leather, ski wax, and outdoor textiles (Kotthoff et al., 2015). Several occupations undergo exposure to alternative PFAS, such as firefighters, ski wax technicians, and PFAS manufacturing workers (Freberg et al., 2010; Plassmann and Berger, 2013; Trowbridge et al., 2020; U.S. EPA 2022a). A significant correlation was determined in ski wax technicians between years worked and serum levels of carboxylic PFAS (Nilsson et al., 2010). In a study of the living areas of fire stations, elevated levels of alternative PFAS in dust samples in the gear locker areas and apparatus bays were detected; further supporting a potential for dermal exposure (Young et al., 2021). Although these alternative PFAS have a shorter half-life in humans they are still very persistent in the environment and can stay for decades to centuries (Cousins et al., 2016), leading to continuous environmental exposure. Additionally, some data show that alternative PFAS are more persistent in the aquatic environment than legacy PFAS (Brendel et al., 2018; Li et al., 2020). Alternative PFAS have been detected in several aquatic environments such as groundwater, surface water, rainwater, and drinking water; in some samples at higher concentrations than legacy PFAS (Goodrow et al., 2020; Kim and Kannan, 2007; Xu et al., 2020).

These data suggest that there is concern for alternative PFAS dermal exposure during swimming or bathing, occupationally, and with consumer product use. Our lab has previously shown that PFOA is absorbed through the skin (Franko et al., 2012) and this leads to functional immune effects (Fairley et al., 2007; Shane et al., 2020). We have also previously shown that an alternative PFAS, PFBA, induces systemic toxicity with dermal exposure similar to that seen with oral PFBA exposure and dermal PFOA exposure (Weatherly et al., 2021). Despite this, no toxicity studies on dermal exposure were identified with PFHxA or PFPeA and only one dermal exposure study was identified with PFHpA (Han et al., 2020).

The present study aims to investigate the systemic effects of subchronic dermal exposure of PFHpA, PFHxA, and PFPeA in a murine model and begin to explore possible mechanisms of resulting toxicity. Due to the detection of these alternative PFAS in humans, the persistence in the environment, and the lack of data from the dermal route of exposure, these experiments are needed to help identify and prioritize PFAS toxicity.

2. Materials and methods

2.1. Animals

Female $B_6C_3F_1$ mice were used in these studies as they are the National Toxicology Program preferred strain for evaluating general toxicity (King-Herbert et al., 2010). 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.

2.2. Test articles and chemicals

Acetone [CAS #67-64-1] and perfluoropentanoic acid (97%, PFPeA) [CAS #2706-90-3] were purchased from Sigma-Aldrich. Perfluoroheptanoic acid (99%; PFHpA) [CAS# 375-85-9], perfluorohexanoic acid (98%; PFHxA) [CAS# 307-24-4], and perfluorooctanoic acid (95%; PFOA) [CAS# 335-67-1] were purchased from Synquest Laboratories. PFAS concentrations were selected based on an initial 7-day range finding study and previous studies conducted with the PFAS PFBA (Weatherly et al., 2021) and the legacy PFAS PFOA (Shane et al., 2020).

2.3. PFAS exposures

For all studies, $B_6C_3F_1$ mice (5/group) were topically treated on the dorsal surface of each ear (25 μl/ear) with vehicle (acetone), PFHpA concentrations (1.25, 2.5, 5% w/v), or PFPeA concentrations (1.25, 2.5, 5% v/v) once a day for 28 consecutive days. As mice were grouped 5 per cage, it is important to mention that there is the possibility of additional oral exposure during grooming. These studies were conducted for the purpose of hazard identification and the concentrations of PFPeA, PFHxA, and PFHpA were based on concentration range finding studies that showed effects in the absence of overt toxicity. Animals were dosed with PFHxA concentrations 2.5, 5, and 10% (v/v) for days 1–4 that was then reduced to 1.25, 2.5, and 5% for days 5–28 due to dermal irritation at the application site. PFOA (0.5% w/v) was used as a comparison to a legacy PFAS, animals were dosed once a day for 28 consecutive days. Body weights were measured daily before exposure to ensure no overt toxicity was occurring due to PFAS exposure. Animals were euthanized by CO_2 asphyxiation approximately 24 h after the last exposure.

2.4. 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 (RNA stabilization solution) 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.

2.5. 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 phosphatase (ALKP), urea nitrogen (BUN), glucose (GLU), and cholesterol (CHOL).

2.6. Analytical PFAS detection

Serum (collected as described above) and urine samples were collected and analyzed for individual PFAS (PFPeA, PFHxA, PFHpA, PFOA) 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). Urine was collected, following excretion from the mouse during euthanasia, in a weigh boat placed under the mouse. Urine samples from each group of mice were pooled and sample volume varied. The results for PFPeA, PFHxA, and PFHpA include the linear isomer only. The results for PFOA include both linear and branched isomers. 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).

2.7. Flow cytometry

Flow cytometry was conducted as previously described (Weatherly et al., 2021). Data was acquired on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo v10 software (TreeStar Inc., Ashland, OR). Cellular populations were defined using the gating strategies outlined in Supplemental Table 1; Fluorescence minus ones (FMOs) were used as gating controls.

2.8. 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 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 β-actin (Taqman). Genes that were evaluated are identified in Supplemental Table 2.

2.9. Histology

Each tissue sample stored in 10% formalin was embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E) and evaluatedby 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).

2.10. Statistical analysis

A one-way analysis of variance (ANOVA) was conducted for analysis of the data generated from the described animal studies. If the ANOVA showed significance at $p \leq 0.05$, the Dunnett's Multiple Range *t*-test was used to compare treatment groups with the control group. Kruskal-Wallis with Dunn's post-test was conducted for Cd36 (PFPeA, PFHxA), Lpl (PFPeA, PFHxA), Acox1 (PFPeA), Ehhadh (PFPeA, PFHxA, PFHpA), Fabp1 (PFPeA, PFHxA, PFHpA), Serpine1 (PFPeA, PFHxA, PFHpA), S100a8 (PFHxA, PFHpA), and Flg (PFHpA) gene expression analysis due to unequal variance. Linear regression analysis was performed on ALKP data (with significance designated by $p < 0.05$). Statistical analysis was performed using Graph Pad Prism version 5.0 (San Diego, CA). Results represent the mean ± SE of 5 mice per group. Statistical significance is designated by $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

3. Results

3.1. 28-day dermal exposure of PFPeA, PFHxA and PFHpA induces significant alterations in both serum and urine PFAS concentrations

A significant increase in PFAS serum concentration for each test article was observed with 5% PFPeA, 2.5% and 5% PFHxA, 1.25%–5% PFHpA, and 0.5% PFOA (Fig. 1A), indicating that there is consequential absorption occurring after PFAS dermal exposure. Statistical analysis could not be performed on urine samples as each concentration is 5 pooled samples with a single data point. Urine PFPeA had the highest percent increase in concentration increasing from 28 μg/mL to 5600 μg/mL with 5%, PFHxA increased in urine from 20 μg/mL to 1700 μg/mL with 5%, and with 5% PFHpA urine concentration increased from 12 μg/mL to 1200 μg/mL. With 0.5% PFOA urine concentrations increased to 260 μg/mL (Fig. 1B).

3.2. PFPeA, PFHxA and PFHpA induce significant alterations in organ weights after 28 days dermal exposure

Similar to what has been reported for previously evaluated PFAS, a statistically significant increase in liver weight (% body weight) was observed following 28-day exposure to PFPeA, PFHxA, and PFHpA (Fig. 2A). Liver weight significantly increased following exposure to 5% PFPeA, 5% PFHxA, and 5% PFHpA (12%, 9% and 18% respectively, vs vehicle-treated mice) (Fig. 2A). However, kidney weights were only significantly increased with 5% PFHpA (Fig. 2B) and thymus weights were significantly increased with PFHpA (2.5%) but not dose dependent and only increased at a single concentration (Fig. 2D). No change in spleen weights (% body weight) were observed with exposure to any of the three PFAS (Fig. 2C). Additionally, no changes in body weight or overt signs of toxicity were observed following 28-days of PFPeA, PFHxA, or PFHpA exposure (Supplemental Fig. 1). PFOA (0.5%) was used as a comparison to a legacy PFAS. A lower PFOA concentration was used as higher PFOA concentrations caused overt toxicity. A significant decrease in body weight was observed after 28-days of 0.5% PFOA exposure (Supplemental Fig. 1). PFOA also induced an increase in liver and kidney weights (% body weight) (120% and 8% increase, respectively) and a decrease in spleen and thymus weights (% body weight) (41% and 71% decrease, respectively) (Supplemental Fig. 2A) (consistent with previously published data).

3.3. PFPeA, PFHxA, and PFHpA altered serum chemistries after dermal exposure

After 28-days of PFAS exposure there was a significant increase in glucose with 5% PFPeA, PFHxA, and PFHpA (30%, 27%, and 30% increase, respectively) compared to the vehicle control (Fig. 3B). A decrease in cholesterol was only observed with 1.25% PFPeA (Fig. 3A). No significant alterations in ALKP were identified via ANOVA, however,

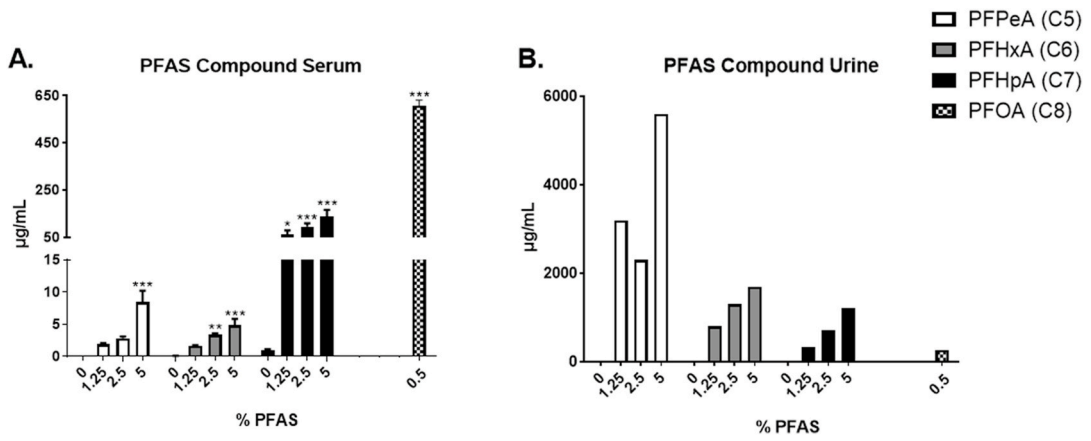

Fig. 1. Changes in PFAS concentration in serum and urine after dermal exposure to PFPeA, PFHxA, or PFHpA. Analysis of changes in the concentration of PFAS in serum samples (A), and concentration of PFAS in urine samples (B) following 28 days of PFAS exposure. Each concentration represents mean (±SE) of 4–5 mice per group. Urine concentrations are 5 pooled samples per group. Statistical significance, relative to 0% vehicle control, was determined by one-way ANOVA followed by Dunnett's post-test indicated as *p < 0.05, **p < 0.01, ***p < 0.001.

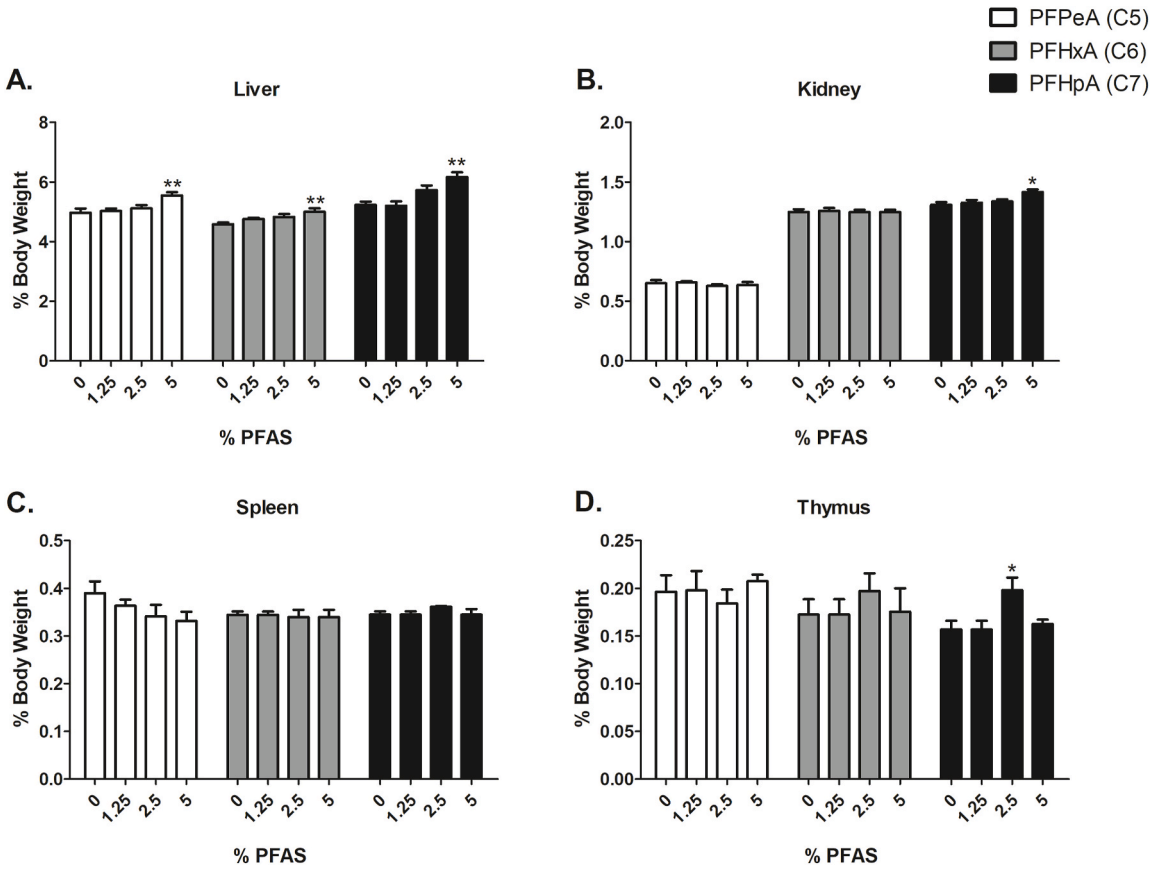

Fig. 2. Changes in organ weights after dermal exposure to PFPeA, PFHxA, or PFHpA. Analysis of changes in liver (A), kidney (B), spleen (C), and thymus (D) weights following 28 days of PFAS exposure. Data is displayed as organ weight as % of body weight. The left kidney was weighed after PFPeA exposure while both kidneys were weighed after PFHxA and PFHpA exposure (B). Each concentration represents mean (±SE) of 4–5 mice per group. Statistical significance, relative to 0% vehicle control, was determined by one-way ANOVA followed by Dunnett's post-test indicated as *p < 0.05, **p < 0.01.

there is a significant linear regression increase (Linear Regression p < 0.05) with PFHpA (Fig. 3C). A significant decrease in urea nitrogen (BUN) was observed with 2.5% and 5% PFPeA (20% and 22%, respectively) and with 5% PFHpA (30% decrease) (Fig. 3D). BUN was unable to be measured with PFHxA due to equipment malfunction. Glucose decreased by 44% and ALKP increased by 112% with 0.5% PFOA (Supp. Fig. 2B). No change was observed with cholesterol or BUN with 0.5% PFOA exposure. No change in alanine aminotransferase (ALT) was observed with PFPeA, PFHpA, and PFOA at any concentration (ALT could not be measured with PFHxA exposure) (data not shown).

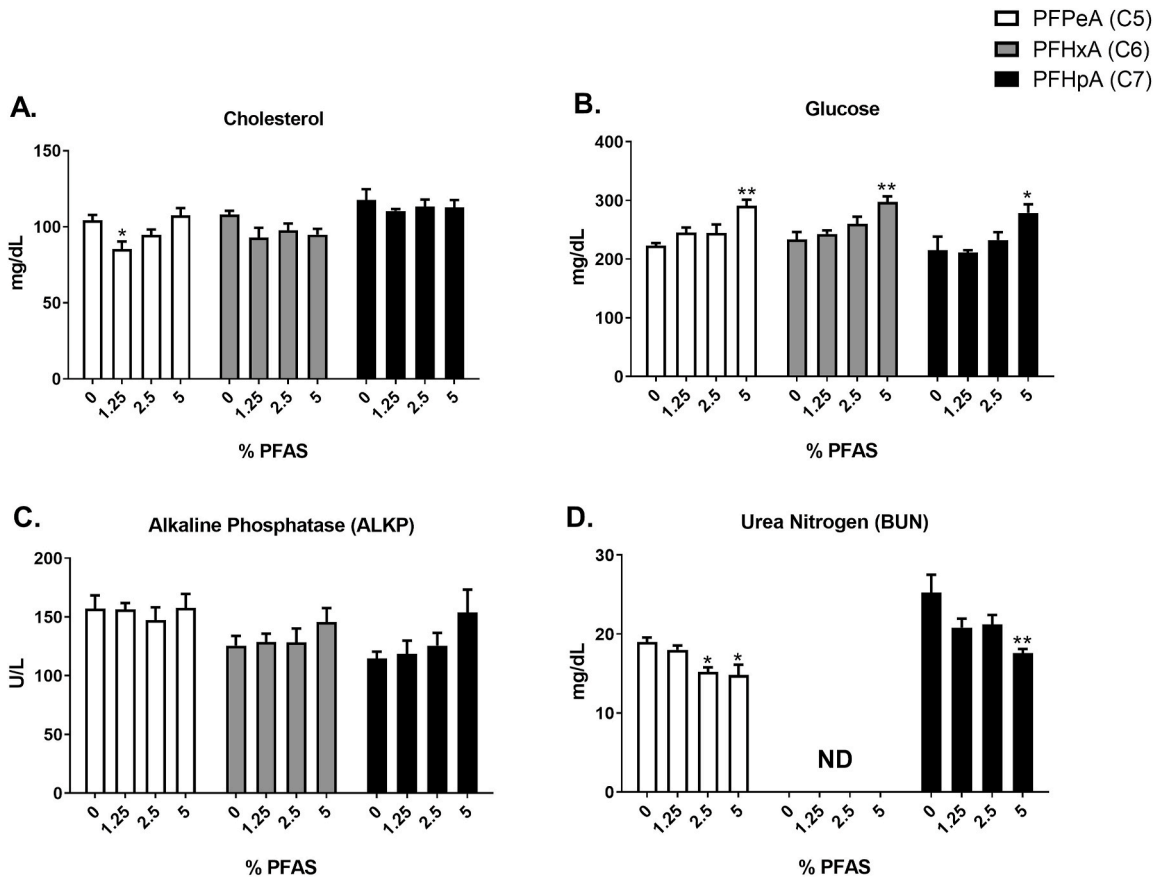

Fig. 3. Changes in serum chemistryafter dermal exposure to PFPeA, PFHxA, or PFHpA. Analysis of changes in cholesterol (A), glucose (B), alkaline phosphatase (ALKP) (C), and urea nitrogen (BUN) (D) following 28 days of PFAS exposure. Each concentration represents mean (±SE) of 4–5 mice per group. BUN was unable to be measured after PFHxA exposure due to equipment malfunction (ND, Not Detected). Statistical significance, relative to 0% vehicle control, was determined by one-way ANOVA followed by Dunnett's post-test indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Significant increase in ALKP was identified via linear regression (Linear Regression $p < 0.05$) with PFHpA.

3.4. Dermal exposure to alternative PFAS for 28 days results in significant immune phenotypic changes

3.4.1. Draining lymph node (dLN)

PFPeA, PFHxA, and PFHpA all increased total cellularity after 28 days of exposure at both 2.5 and 5% in the dLN (Table 1). In the dLN, significant increases were also observed in both frequency and cell number of B-cells with PFPeA (2.5% and 5%), suggesting a potential preferential expansion of B-cells. PFHpA also induced an increase in B-cells with 2.5% and 5% but only in cell number. $CD4^+$ and $CD8^+$ T-cell number increased with PFPeA (1.25%, 2.5%, 5%), PFHxA (2.5%, 5%) and PFHpA (2.5%, 5%). NK cell number increased with PFPeA (2.5%, 5%), PFHxA (2.5%, 5%), and PFHpA (5%), while an increase in NK cell frequency was only observed with 2.5% PFHxA. An increase in both eosinophil cell number and frequency occurred with 5% PFPeA and 2.5% PFHpA. Total dendritic cell (DC) number and frequency increased with PFHxA exposure and DC number increased with PFPeA and PFHpA exposure. CD11b + cells increased in both frequency and number with 2.5% PFHxA and 5% PFHpA. MHCII (activation marker) MFI on B-cells was also significantly increased with PFPeA (2.5%, 5%), PFHxA (5%), and PFHpA (2.5%, 5%) and CD86 MFI (activation marker) on B-cells increased with PFHxA (5%) and PFHpA (2.5%, 5%). MHCII and CD86 MFI on DCs decreased with both PFPeA (5%) and PFHpA (5%) but decreased with PFHxA (2.5%) (Table 1). In contrast, $CD4^+$ T-cells decreased in both cell number and frequency with 0.5% PFOA (Supplemental Table 3). PFOA also induced a decrease in eosinophil cell number and MHCII MFI on B-cells (Supplemental Table 3).

3.4.2. Skin

Phenotypic analysis of the skin resulted in increases in frequency of $CD45^+$ cells following 28 days of exposure with 2.5% and 5% PFPeA and PFHxA (Table 2). Frequency of $CD4^+$ T-cells (PFPeA 1.25%, 2.5%, 5%; PFHxA and PFHpA 2.5%, 5%), $CD8^+$ T-cells (PFPeA 2.5%, 5%; PFHpA 5%), NK cells (PFHxA 5%), eosinophils (PFPeA, PFHxA, PFHpA 2.5%, 5%), neutrophils (PFPeA, PFHxA, PFHpA 5%), dendritic cells (PFPeA, PFHpA 2.5%, 5%), and CD11b + F4/80+ DCs (PFPeA 1.25%, 2.5%, 5%; PFHpA 2.5%, 5%) significantly increased after 28-day exposure (Table 2). Frequency of CD11b- F4/80+ DCs decreased with PFHxA (5%) but increased with PFHpA (2.5%, 5%) and CD11b- F4/80- DCs increased with PFPeA (2.5%, 5%) and decreased with PFHxA and PFHpA (1.25%). Statistically significant increases were also observed in the absolute number of total cells (PFPeA 5%), $CD45^+$ cells (PFPeA and PFHxA 5%), $CD4^+$ (PFPeA and PFHxA 2.5%, 5%; PFHpA 5%), $CD8^+$ T-cells (PFPeA and PFHpA 5%; PFHxA 2.5%, 5%), NK cells (PFPeA and PFHxA 5%), eosinophils (PFPeA and PFHxA 2.5%, 5%; PFHpA 5%), neutrophils (PFPeA, PFHxA, and PFHpA 5%), total dendritic cells (PFPeA, PFHxA, and PFHpA 5%), CD11b- F4/80+ DCs (PFPeA and PFHpA 5%) and CD11b + F4/80+ DCs (PFPeA 2.5%, 5%; PFHxA and PFHpA 5%). Interestingly, in contrast to the overall increases in cells observed with the alternative PFAS, 0.5% PFOA significantly decreased frequency and cell number in $CD45^+$ cells, eosinophils, total DCs, CD11b- F/480+ DCs, CD11b + F4/80+ DCs, and CD11b- F4/80 DCs (Supplemental Table 4).

3.4.3. Spleen

Lesser changes were observed in the spleen with the alternative

Table 1
Draining lymph node phenotyping of mice dermally exposed to PFAS.

dLN	28 days											
	C5; PFPeA (v/v)				C6; PFHxA (v/v)				C7; PFHpA (w/v)			
Parameter	0%	1.25%	2.5%	5%	0%	1.25%	2.5%	5%	0%	1.25%	2.5%	5%
Total Cellularity (x 10^6)	2.93 ± 0.29	6.15 ± 0.57	**8.45 ± 1.43****	**10.50 ± 0.93*****	13.1 ± 1.52	15.00 ± 1.92	**20.30 ± 1.81***	**21.70 ± 1.26****	3.07 ± 0.45	4.05 ± 0.31	**5.97 ± 1.06***	**7.78 ± 0.90****
$CD4^+$ (x 10^6)	1.30 ± 0.10	**2.63 ± 0.27***	**3.34 ± 0.55****	**3.99 ± 0.36*****	5.41 ± 0.61	5.93 ± 0.59	**8.16 ± 0.58***	**8.99 ± 0.49****	1.30 ± 0.20	1.77 ± 0.17	**2.47 ± 0.44***	**3.03 ± 0.31****
$CD4^+$ (%)	44.32 ± 0.26	42.52 ± 0.79	**39.54 ± 0.62*****	**38.02 ± 0.95*****	41.33 ± 1.08	40.24 ± 1.70	40.56 ± 1.03	41.58 ± 0.62	42.32 ± 0.40	43.44 ± 1.00	41.54 ± 0.58	**39.24 ± 0.83***
$CD8^+$ (x 10^5)	8.61 ± 0.58	**18.60 ± 0.18***	**25.00 ± 3.80*****	**30.20 ± 0.23*****	37.3 ± 4.13	42.50 ± 4.15	**56.80 ± 5.72***	**58.40 ± 4.46***	8.46 ± 0.14	11.50 ± 0.97	**17.20 ± 3.10***	**22.10 ± 2.59****
$CD8^+$ (%)	29.48 ± 0.63	30.24 ± 0.44	29.96 ± 0.86	28.88 ± 0.40	28.60 ± 0.71	28.94 ± 1.29	27.94 ± 0.36	26.82 ± 0.64	27.34 ± 0.78	28.26 ± 0.62	28.6 ± 0.62	28.36 ± 0.20
B-cells (x 10^5)	3.64 ± 0.46	8.67 ± 0.72	**12.60 ± 2.36****	**17.00 ± 1.58*****	23.3 ± 1.60	28.40 ± 6.63	32.50 ± 3.50	37.30 ± 1.54	4.29 ± 0.52	5.40 ± 0.44	**8.69 ± 0.16***	**11.50 ± 1.39*****
B-cells (%)	12.26 ± 0.63	14.18 ± 0.59	**15.00 ± 0.92***	**16.22 ± 0.70****	18.13 ± 1.00	18.06 ± 2.12	15.94 ± 0.38	17.38 ± 0.94	14.12 ± 0.76	13.44 ± 1.00	14.86 ± 0.84	14.70 ± 0.13
NK (x 10^4)	1.55 ± 0.14	3.23 ± 0.30	**5.52 ± 0.71*****	**7.50 ± 0.56*****	8.45 ± 1.04	11.60 ± 0.64	**19.50 ± 2.26****	**17.70 ± 2.11****	1.84 ± 0.29	1.84 ± 0.21	3.23 ± 0.68	**4.78 ± 0.67*****
NK (%)	0.55 ± 0.07	0.53 ± 0.03	0.69 ± 0.07	0.73 ± 0.05	0.65 ± 0.06	0.82 ± 0.08	0.95 ± 0.03*	0.80 ± 0.06	0.60 ± 0.03	**0.45 ± 0.03***	0.53 ± 0.03	0.61 ± 0.04
Eosinophils (x 10^2)	22.10 ± 2.13	20.90 ± 7.02	**81.90 ± 15.70***	**140.00 ± 23.00*****	75.10 ± 13.20	96.70 ± 17.60	140.00 ± 17.30	142.00 ± 22.90	5.11 ± 1.07	7.53 ± 1.81	**20.40 ± 3.85***	**22.80 ± 6.04***
Eosinophils (% x 10^{-2})	7.68 ± 0.77	3.58 ± 1.13	9.88 ± 1.08	**13.22 ± 1.81***	5.70 ± 0.62	6.40 ± 0.64	6.94 ± 0.71	6.40 ± 0.84	1.73 ± 0.36	1.80 ± 0.41	**3.42 ± 0.22***	2.68 ± 0.58
Neutrophils (x 10^2)	9.18 ± 2.19	12.30 ± 1.75	19.10 ± 3.38	**22.40 ± 5.58***	20.60 ± 3.18	18.80 ± 5.25	21.50 ± 1.28	16.70 ± 2.99	9.33 ± 2.03	8.55 ± 1.38	9.51 ± 1.88	**2.18 ± 4.16*****
Neutrophils (% x 10^{-2})	3.06 ± 0.62	2.12 ± 0.41	2.36 ± 0.35	**2.20 ± 0.60**	1.59 ± 0.22	1.19 ± 0.23	1.08 ± 0.07	**0.76 ± 0.12***	3.12 ± 0.62	2.09 ± 0.32	**1.56 ± 0.07***	2.68 ± 0.33
Dendritic Cells (x 10^4)	3.29 ± 0.31	6.24 ± 0.42	**10.40 ± 1.31*****	**12.60 ± 1.09*****	13.30 ± 1.43	29.00 ± 2.99	**47.00 ± 5.39*****	**35.40 ± 5.48****	9.10 ± 1.78	15.60 ± 6.03	9.50 ± 1.42	**18.20 ± 3.38***
Dendritic Cells (%)	1.13 ± 0.07	1.02 ± 0.03	1.31 ± 0.15	1.21 ± 0.08	1.04 ± 0.12	**2.01 ± 0.22****	**2.32 ± 0.14*****	1.60 ± 0.19	2.90 ± 0.43	4.23 ± 1.95	1.70 ± 0.17	2.28 ± 0.20
CD11b+ (x 10^4)	5.01 ± 0.46	7.21 ± 1.61	**17.00 ± 4.33***	**24.00 ± 3.48*****	11.10 ± 2.61	16.20 ± 3.71	**25.30 ± 2.29***	**26.00 ± 3.54***	2.57 ± 0.40	3.18 ± 0.81	6.87 ± 1.47	**13.40 ± 1.96*****
CD11b+ (%)	1.71 ± 0.05	1.26 ± 0.29	1.92 ± 0.19	2.26 ± 0.17	0.82 ± 0.09	1.04 ± 0.12	**1.25 ± 0.04***	1.18 ± 0.11	0.83 ± 0.04	0.74 ± 0.16	1.09 ± 0.09	**1.69 ± 0.10*****
MHCII B-cells (MFI x 10^2)	85.80 ± 6.41	105.00 ± 1.83	**115.00 ± 6.25****	**126.00 ± 6.53*****	5.84 ± 0.61	7.07 ± 0.37	7.16 ± 0.11	**8.02 ± 0.64***	24.80 ± 1.57	33.30 ± 3.62	**44.30 ± 4.02*****	**43.10 ± 1.85****
MHCII DCs (MFI x 10^2)	241.00 ± 10.30	211.00 ± 13.10	200.00 ± 18.20	**170.00 ± 14.10****	45.30 ± 3.36	57.30 ± 2.79	**63.50 ± 5.71***	46.30 ± 2.97	45.40 ± 2.36	47.00 ± 3.13	53.40 ± 2.49	**58.90 ± 2.65****
CD86 B-cells (MFI)	1015.20 ± 60.19	1140.40 ± 46.06	1131.40 ± 96.26	1188.80 ± 47.41	80.63 ± 5.92	98.74 ± 6.64	93.54 ± 1.71	101.54 ± 3.64	710.00 ± 41.28	851.20 ± 55.68	**908.40 ± 44.09***	**879.80 ± 40.32***
CD86 DCs (MFI x 10^2)	43.90 ± 2.46	42.60 ± 1.44	40.30 ± 1.05	**36.80 ± 2.23***	7.58 ± 0.36	11.60 ± 1.51	**13.20 ± 1.29****	9.15 ± 0.71	23.10 ± 1.03	22.90 ± 1.99	25.10 ± 0.72	**28.50 ± 0.78***

Values are expressed as the mean (±SE) for each group (n = 5 mice/group).
*p < 0.05, **p < 0.01, ***p < 0.001.

PFAS, showing a significant decrease in the frequency of B-cells (PFHpA 5%), $CD8^+$ T-cells (PFHpA 1.25%), eosinophils (PFHpA 1.25%), CD11b + Ly6C + cells (PFHxA 2.5%, 5%), and an increase in NK cell frequency (PFPeA 1.25%, 2.5%, 5%; PFHxA 2.5%; PFHpA 5%) and total DCs (PFHpA 5%) (Supp Table 5). A significant decrease was observed in number of $CD4^+$ T-cells (PFPeA 2.5%), CD11b + Ly6C- cells (PFPeA 2.5%), and an increase in number of NK cells (PFHpA 5%) (Supp. Table 5). An increase in MFI of MHCII was observed on B-cells (PFPeA and PFHpA 2.5%, 5%) and of CD86 on B-cells (PFPeA and PFHxA 2.5%, 5%; PFHpA 1.25%, 2.5%, 5%) (Supp. Table 5). The legacy PFOA control showed much more pronounced changes in the spleen. A significant decrease was observed in total cell number, number of B-cells, $CD4^+$ T-cells, NK cells, eosinophils, total DCs, CD11b + cells, CD11b + Ly6C + cells, and CD11b + Ly6C- cells with 0.5% PFOA (Supp. Table 6). PFOA also induced an increase in frequency of $CD4^+$ and $CD8^+$ T-cells and neutrophils, and a decrease in frequency in NK cells, CD11b + cells, CD11b + Ly6C + cells, and CD11b + Ly6C- cells along with a decrease in MFI of MHCII on B-cells and DCs (Supplemental Table 6).

3.5. Dermal exposure to PFAS for 28 days results in histopathological changes in the liver and skin

In the liver, histopathological examination revealed that PFPeA, PFHxA, and PFHpA induced hepatocellular hypertrophy affecting the centrilobular region. PFPeA induced minimal hypertrophy at 1.25% (5/5 mice) and mild hypertrophy at 2.5% (5/5 mice) and 5% (5/5 mice) (Table 3). Individual animals treated PFHxA showed minimal hypertrophy at 1.25% (2/5 mice), 2.5% (5/5 mice) and 5% (5/5 mice) (Table 3). PFHpA exposed mice exhibited minimal hypertrophy at 1.25% (3/5 mice), 2.5% (5/5 mice), and 5% (2/5 mice) and mild hypertrophy at 5% (3/5 mice) (Table 3). Hepatocellular hypertrophy was characterized by increased cytoplasmic eosinophilia, decreased glycogen content, and increased cellular volume of hepatocytes in centrilobular locations. No histopathological changes were observed in the liver with 0% PFPeA, PFHxA or PFHpA vehicle control. Similarly, 0.5% PFOA exposure altered liver morphology by inducing hepatocellular hypertrophy presenting as a diffuse change affecting all zones of the liver (Supp. Table 7). Mice exposed to 0.5% PFOA showed a marked changed in hypertrophy (diffuse) in 5/5 mice. Lipid droplets and steatosis were not reported by the pathologist.

Table 2
Skin phenotyping of mice dermally exposed to PFAS.

Ear	28 days											
	C5; PFPeA (v/v)				C6; PFHxA (v/v)				C7; PFHpA (w/v)			
Parameter	0%	1.25%	2.5%	5%	0%	1.25%	2.5%	5%	0%	1.25%	2.5%	5%
Total Cellularity (x 10^6)	0.83 ± 0.06	1.05 ± 0.10	0.88 ± 0.04	**1.41 ± 0.11****	0.81 ± 0.09	0.83 ± 0.09	0.91 ± 0.05	0.99 ± 0.11	1.00 ± 0.09	0.96 ± 0.08	0.97 ± 0.05	1.19 ± 0.08
$CD45^+$ (x 10^4)	6.09 ± 0.88	8.61 ± 1.11	10.30 ± 1.26	**19.40 ± 2.55****	5.93 ± 0.78	7.21 ± 1.13	9.82 ± 0.79	**11.50 ± 2.03***	12.60 ± 0.80	8.01 ± 1.58*	11.00 ± 1.57	14.60 ± 0.67
$CD45^+$ (%)	7.21 ± 0.68	8.16 ± 0.67	**11.46 ± 1.03****	**13.60 ± 0.97****	7.34 ± 0.670	8.51 ± 0.85	**10.91 ± 1.00***	**11.22 ± 1.00***	12.92 ± 1.42	8.04 ± 1.11	11.32 ± 1.45	12.38 ± 0.81
$CD4^+$ (x 10^3)	1.19 ± 0.23	2.55 ± 0.41	**4.65 ± 0.67****	**10.80 ± 1.17****	1.63 ± 0.21	2.40 ± 0.36	**5.01 ± 0.60****	**6.11 ± 1.17****	2.30 ± 0.11	1.49 ± 0.38	4.31 ± 1.02	**6.06 ± 0.33****
$CD4^+$ (%)	1.95 ± 0.27	**2.93 ± 0.23***	**4.47 ± 0.14****	**5.64 ± 0.27****	2.75 ± 0.11	3.34 ± 0.09	**5.05 ± 0.25****	**5.29 ± 0.32****	1.85 ± 0.12	1.78 ± 0.19	**3.70 ± 0.93****	**4.18 ± 0.24****
$CD8^+$ (x 10^2)	0.86 ± 0.02	1.38 ± 0.17	2,79 ± 0.39	**8.33 ± 1.57****	0.66 ± 0.16	3.38 ± 2.57	**1.91 ± 0.23***	**2.15 ± 0.46****	1.11 ± 0.16	0.79 ± 0.14	1.77 ± 0.27	**2.81 ± 0.39****
$CD8^+$ (%)	0.14 ± 0.02	0.16 ± 0.02	**0.27 ± 0.03***	**0.42 ± 0.04****	0.12 ± 0.03	0.48 ± 0.37	0.19 ± 0.02	0.20 ± 0.04	0.09 ± 0.01	0.11 ± 0.03	0.16 ± 0.01	**0.20 ± 0.03***
NK (x 10^2)	3.54 ± 0.57	5.64 ± 0.85	5.86 ± 0.63	**16.10 ± 4.27****	3.72 ± 0.47	4.76 ± 0.96	6.45 ± 0.60	**9.94 ± 2.21****	7.28 ± 0.60	3.89 ± 0.91	7.43 ± 1.69	7.96 ± 0.70
NK (%)	0.58 ± 0.05	0.66 ± 0.06	0.57 ± 0.01	0.80 ± 0.12	0.63 ± 0.01	0.66 ± 0.07	0.66 ± 0.04	**0.85 ± 0.09***	0.58 ± 0.03	0.48 ± 0.04	0.66 ± 0.08	0.56 ± 0.07
Eosinophils (x 10^3)	1.18 ± 0.24	2.72 ± 0.51	**5.85 ± 1.03***	**13.50 ± 2.05****	1.87 ± 0.30	2.77 ± 0.56	**7.42 ± 0.74****	**9.87 ± 1.78****	2.55 ± 0.30	1.41 ± 0.42	5.54 ± 1.71	**6.45 ± 1.12***
Eosinophils (%)	1.92 ± 0.18	3.13 ± 0.31	**5.57 ± 0.42****	**7.35 ± 1.24****	3.09 ± 0.13	3.82 ± 0.34	**7.59 ± 0.65****	**8.60 ± 0.38****	2.06 ± 0.27	1.60 ± 0.26	**4.57 ± 0.96***	**4.36 ± 0.63***
Neutrophils (x 10^2)	1.79 ± 0.33	2.28 ± 0.53	3.16 ± 0.37	**21.60 ± 8.98***	0.85 ± 0.14	1.18 ± 0.22	4.94 ± 1.24	**13.00 ± 3.65****	2.82 ± 0.44	1.59 ± 0.24	2.48 ± 0.43	**7.10 ± 1.15****
Neutrophils (%)	0.33 ± 0.08	0.27 ± 0.06	0.32 ± 0.05	**0.97 ± 0.31***	0.14 ± 0.01	0.17 ± 0.02	0.51 ± 0.13	**1.13 ± 0.23****	0.22 ± 0.02	0.21 ± 0.02	0.24 ± 0.04	**0.49 ± 0.07****
Dendritic Cells (x 10^3)	3.55 ± 0.47	6.78 ± 0.97	9.44 ± 2.00	**20.20 ± 2.65****	5.22 ± 0.67	4.99 ± 0.68	9.09 ± 0.72	**10.10 ± 1.77***	4.02 ± 0.23	2.18 ± 0.47	4.75 ± 0.88	**7.25 ± 0.31****
Dendritic Cells (%)	5.87 ± 0.23	7.83 ± 0.37	**8.97 ± 0.96****	**10.42 ± 0.36****	9.15 ± 1.15	7.09 ± 0.33	9.30 ± 0.47	8.88 ± 0.39	3.21 ± 0.11	2.69 ± 0.15	**4.21 ± 0.26****	**4.99 ± 0.15****
CD11b- F4/80+ DCs (x 10^2)	11.70 ± 1.59	17.60 ± 3.16	17.80 ± 3.48	**45.60 ± 8.63****	6.89 ± 1.55	5.74 ± 0.72	6.58 ± 0.67	4.22 ± 0.49	6.31 ± 0.40	5.44 ± 0.94	7.95 ± 0.98	**14.50 ± 0.97****
CD11b- F4/80+ DCs (%)	1.94 ± 0.13	2.10 ± 0.37	1.73 ± 0.22	2.36 ± 0.35	1.24 ± 0.28	0.87 ± 0.14	0.67 ± 0.05	**0.39 ± 0.03****	0.51 ± 0.03	0.70 ± 0.05	**0.75 ± 0.08***	**1.00 ± 0.07****
CD11b + F4/80+ DCs (x 10^2)	11.10 ± 1.40	26.50 ± 4.08	**44.50 ± 9.79***	**86.70 ± 10.20****	19.20 ± 2.86	22.30 ± 3.03	35.80 ± 2.66	**48.30 ± 7.74****	7.53 ± 0.54	4.52 ± 0.98	13.00 ± 2.97	**18.90 ± 1.84****
CD11b + F4/80+ DCs (%)	1.85 ± 0.08	3.06 ± 0.19*	**4.22 ± 0.51****	**4.51 ± 0.18****	3.41 ± 0.54	3.18 ± 0.24	3.62 ± 0.16	4.32 ± 0.30	0.61 ± 0.05	0.56 ± 0.02	**1.13 ± 0.12****	**1.31 ± 0.14****
CD11b- F4/80- DCs (x 10^3)	1.21 ± 0.18	2.15 ± 0.38	2.79 ± 0.54	**6.19 ± 0.94****	1.74 ± 0.20	1.47 ± 0.24	2.82 ± 0.24	2.90 ± 0.54	2.58 ± 0.20	**1.12 ± 0.29****	2.42 ± 0.45	3.38 ± 0.19
CD11b- F4/80- DCs (%)	1.99 ± 0.08	2.44 ± 0.16	**2.64 ± 0.20***	**3.16 ± 0.10****	3.05 ± 0.33	**2.05 ± 0.05****	2.89 ± 0.19	2.51 ± 0.14	2.05 ± 0.10	**1.34 ± 0.16****	2.15 ± 0.12	2.32 ± 0.03

Values are expressed as the mean (±SE) for each group (n = 5 mice/group).
*p < 0.05, **p < 0.01, ***p < 0.00.

Dose-related changes were observed in multiple layers of the skin with all three alternative PFAS (Table 3). Minimal epidermal hyperplasia (increased number (3–4) layers of keratinocytes with increased hyaline granules in the severely affected areas) was observed with PFPeA at 2.5% (4/5 mice) and 5% (2/5 mice), with PFHxA at 1.25% (5/5 mice) and 2.5% (5/5 mice) and 5% (1/5 mice), and with PFHpA at 2.5% (2/5 mice) and 5% (4/5 mice). Mild epidermal hyperplasia (5–6 layers of keratinocytes) occurred with PFPeA (5% 3/5 mice), PFHxA (5% 4/5 mice), and PFHpA (5% 1/4 mice) (Table 4). PFPeA and PFHpA exposure showed only minimal hyperkeratosis (characterized by increased amounts of compacted keratin) with 2/5 mice (5% PFPeA) and 4/5 mice (5% PFHpA). PFHxA showed both minimal (1.25% 1/5 mice, 2.5% 4/5 mice, and 5% 2/5 mice) and mild (5% 3/5 mice) hyperkeratosis (Table 3). Minimal dermal mixed cell inflammation (characterized by neutrophils, lymphocytes, and/or macrophages scattered within the fibrosis) was observed with PFPeA (2.5% 2/5 mice and 5% 4/5 mice), PFHxA (5% 4/5 mice), and PFHpA (5% 1/5 mice). Dermal mononuclear cell inflammation was only observed with 5% PFHpA (1/5 mice). PFPeA and PFHxA exposure both at 5% induced minimal dermal focal fibrosis (4/5 mice and 5/5 mice, respectively) (Table 4). Focal cartilage degeneration/regeneration only occurred with 5% PFHpA in 1/5 mice and no epidermal necrosis was observed with any alternative PFAS exposure (Table 3). Irritation at the site of application resulted from exposure of all three alternative PFAS. Interestingly, no irritation was observed with 0.5% PFOA. No histopathological changes were seen in the skin within any of the 0% control groups (acetone). In the ear, 0.5% PFOA only induced minimal hyperplasia (1/5 mice) and minimal dermal mononuclear cell inflammation (1/5 mice) (Supplemental Table 7). No PFPeA, PFHxA, or PFHpA-related microscopic observations were made in the kidney or the spleen (data not shown). With 0.5% PFOA, decreased lymphocyte cellularity was observed in the spleen in 1/5 mice (minimal) and 2/5 mice (mild) (Supplemental Table 7).

Gene expression in the liver was investigated based on two PCR pathway-based arrays, peroxisome proliferator-activated receptor (PPAR) targets and hepatoxicity, previously used to investigate effects of PFBA exposure (Weatherly et al., 2021). A majority of the significant responses showed a dose-dependent trend, for both liver and skin. Genes involved in steatosis (*Cd36, Lpl*), phospholipidosis (*Fabp1*), necrosis (*Serpine1*), adipogenesis (*Lpl*), fatty acid metabolism (*Pltp, Acox1, Ehhadh*), lipid transport (*Lpl*), and PPAR ligand transport (*Cd36*) were upregulated (Fig. 4). Increases in *Cd36* were observed with PFPeA (5%, 6.5-fold), PFHxA (5%, 4.6-fold), and PFHpA (2.5%, 4-fold and 5%,

Table 3
Incidence and degree of hepatocyte and epidermal injury following dermal exposure to PFAS in mice.

Parameter	C5; PFPeA (v/v)				C6: PFHxA (v/v)				C7; PFHpA (w/v)			
	0%	1.25%	2.5%	5%	0%	1.25%	2.5%	5%	0%	1.25%	2.5%	5%
Liver												
Hypertrophy, centrilobular, hepatocyte												
Minimal	0	5	0	0	0	2	5	5	0	3	5	2
Mild	0	0	5	5	0	0	0	0	0	0	0	3
Ear												
Hyperplasia, epidermis, focal												
Minimal	0	0	4	2	0	5	5	1	0	0	2	4
Mild	0	0	0	3	0	0	0	4	0	0	0	1
Hyperkeratosis, epidermis, focal												
Minimal	0	0	0	2	0	1	4	2	0	0	0	4
Mild	0	0	0	0	0	0	0	3	0	0	0	0
Necrosis, epidermis, multifocal												
Minimal	0	0	0	0	0	0	0	0	0	0	0	0
Inflammation, mixed cell, dermis												
Minimal	0	0	2	4	0	0	0	4	0	0	0	1
Inflammation, mononuclear cell, dermis												
Minimal	0	0	0	0	0	0	0	0	0	0	0	1
Fibrosis, dermis, focal												
Minimal	0	0	0	4	0	0	0	5	0	0	0	0
Degeneration/regeneration, cartilage, focal												
Mild	0	0	0	0	0	0	0	1	0	0	0	0

Each dose group of mice contained 5 mice total.
Dermal PFPeA, PFHxA, and PFHpA exposure results in changes in gene expression in the liver and skin.

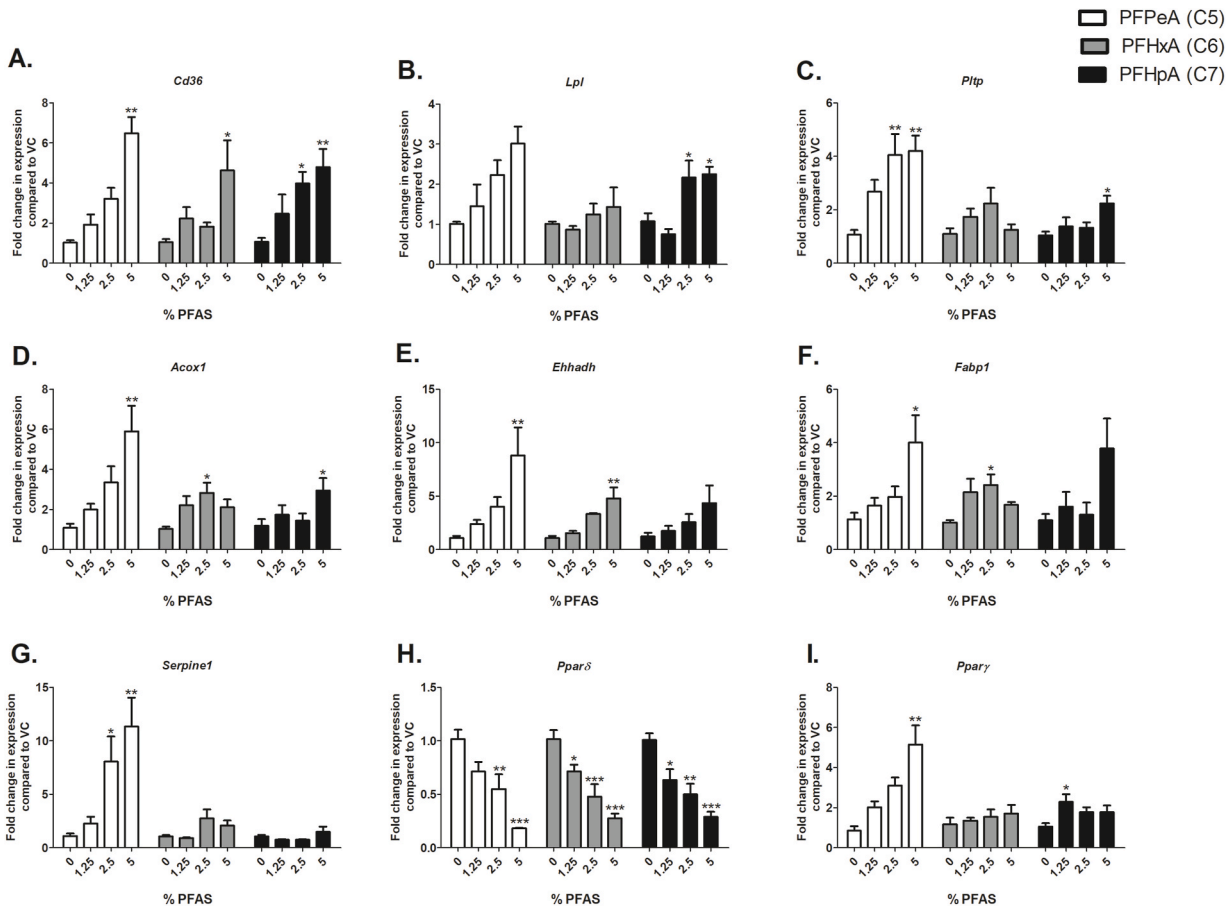

Fig. 4. Liver gene expression following dermal exposure to PFPeA, PFHxA, or PFHpA. Gene expression in the liver following 28 days of PFAS exposure. Changes in *Cd36* (A), *Lpl* (B), *Pltp* (C), *Acox1* (D), *Ehhadh* (E), *Fabp1* (F), and *Serpine1* (G) were evaluated. Data shown as the mean (±SE) of 5 mice per group. Statistical significance, relative to 0% vehicle control (VC), was determined by one-way ANOVA with Dunnett's post-test where *$p < 0.05$, **$p < 0.01$. Kruskal-Wallis with Dunn's post-test was conducted for *Cd36* (PFPeA, PFHxA), *Lpl* (PFPeA, PFHxA), *Acox1* (PFPeA), *Ehhadh* (PFPeA, PFHxA, PFHpA), *Fabp1* (PFPeA, PFHxA, PFHpA), and *Serpine1* (PFPeA, PFHxA, PFHpA) due to unequal variance.

4.8-fold) (Fig. 4A). Gene expression of *Lpl* increased with 2.5% PFHpA (2.2-fold) and 5% PFHpA (2.3-fold) (Fig. 4B). An increase in *Pltp* was demonstrated with PFPeA (2.5% and 5%) and PFHpA (5%) (Fig. 4C) and in *Acox1* with PFPeA (5%), PFHxA (2.5%), and PFHpA (5%) (Fig. 4D). *Ehhadh* increased with 5% PFPeA (8.8-fold) and 5% PFHxA (4.8-fold) (Fig. 4E) and *Fabp1* increased with 5% PFPeA and 2.5% PFHxA (Fig. 4F). The most significant increase with an alternative PFAS occurred with PFPeA exposure in *Serpine1* expression with 2.5% (8-fold) and 5% (11.3-fold) (Fig. 4G). PPAR isoforms were also evaluated, with *Pparδ* expression decreasing with PFPeA (2.5% and 5%), PFHxA (1.25%, 2.5%, and 5%), and PFHpA (1.25%, 2.5%, and 5%) exposure (Fig. 4H) and *Pparγ* expression increasing with 5% PFPeA and 1.25% PFHpA exposure (Fig. 4I). No changes were observed in *Pparα* in the liver (data not shown). PFOA induced an increase in gene expression in *Cd36* (12.2-fold), *Lpl* (13.1-fold), *Ehhadh* (57.7-fold), and *Serpine1* (10.5-fold) and a decrease in gene expression in *Pparδ* with 0.5% exposure (Supplemental Fig. 2C).

The dermal toxicity mechanism was further evaluated with investigations of gene expression in the skin. Inflammatory cytokine *Il6* significantly increased with 5% PFPeA (2.5-fold) (Fig. 5A). The Th2 skewing cytokine, *Tslp*, also increased with 5% PFPeA exposure (3-fold) and 5% PFHpA (5.1-fold) (Fig. 5B). Gene expression of *Serpine1*, involved in necrosis, increased at 5% PFPeA (3-fold) and 5% PFHpA (1.7-fold) (Fig. 5D). Expression of both danger-associated molecular patterns (DAMPs) *S100a7a* and *S1008a* increased, with *S100a7a* having the largest increase with 5% PFPeA exposure (42.5-fold) and *S100a8* increasing with 5% PFHxA (16.8-fold) and 5% PFHpA (6.7-fold) (Fig. 5C, E). Interestingly, *PPARα* decreased at both 5% PFPeA and PFHxA but there was no significant decrease with PFHpA exposure (Fig. 5F). *Pparγ* gene expression decreased at 1.25%, 2.5%, and 5% PFPeA and 2.5% and 5% with PFHxA and PFHpA (Fig. 5H), while no changes were observed in *Pparδ* (Fig. 5G). PFOA induced a significant increase with *Serpine1*, a slight significant increase in *Pparδ,* and a significant decrease in *Pparγ* (Supp. Fig.2D). Multiple changes were observed with 3 skin barrier integrity genes (Fig. 6). PFHxA and PFHpA at 5% exposure increased *Lor* gene expression (Fig. 6A), *Flg* increased with PFPeA (5%) and PFHxA and PFHpA (2.5%, 5%) (Fig. 6B), and *Krt10* increased with PFPeA (1.25%, 2.5%, 5%) and PFHpA (2.5%, 5%) (Fig. 6C). The only skin barrier integrity gene that increased with 0.5% PFOA was *Flg* (Supp. Fig. 2D).

4. Discussion

The skin is the largest organ in the body and provides an important role as a physical barrier and as an immunologically active environment. Disruptions to the skin barrier by dermal chemical exposure can lead to inflammation, allergy, and immunotoxicity. Dermal exposure is a major occupational concern as the CDC estimates millions of workers in the US are exposed to dermally absorbable chemicals (Anderson and Meade, 2014; NORA, 2019). Investigations into PFAS dermal exposure, especially regarding alternative PFAS, are lacking even though exposure to these widely used chemicals is a growing concern both for the public and for workers. These studies attempted to fill in some of the data gaps by investigating the systemic toxicity and skin absorption of three occupationally relevant carboxylic PFAS (PFPeA, PFHxA, and PFHpA) following dermal exposure in a murine model.

Similar to PFOA and PFOS, all three PFAS exposures induced an increase in liver weight (Fig. 2A) and hepatocellular hypertrophy

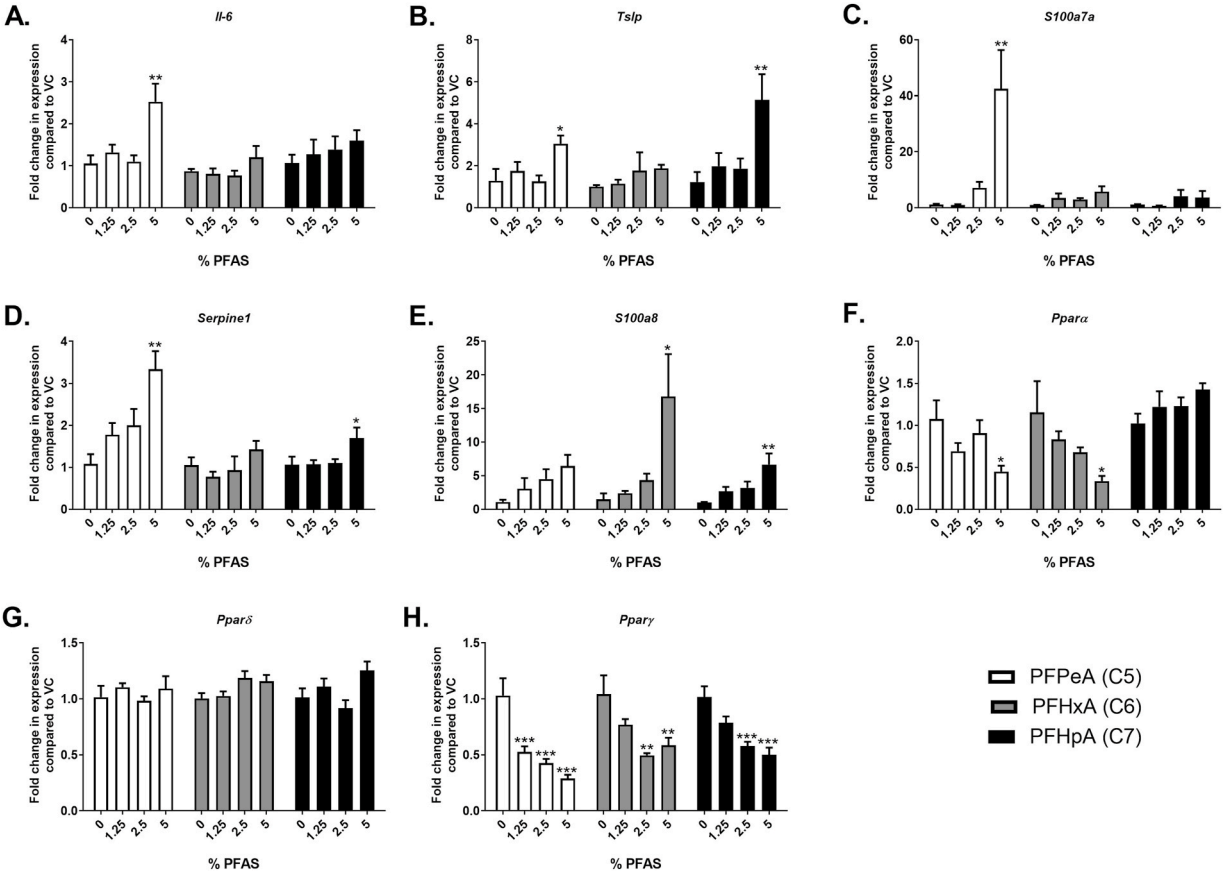

Fig. 5. Skin gene expression following dermal exposure to PFPeA, PFHxA, or PFHpA. Gene expression in the ear following 28 days of PFAS exposure. Changes in *Il-6* (A), *Tslp* (B), *Serpin1* (C), *S100a7a* (D), *S100a8* (E), and *Ppara* (F) were evaluated. Data shown as the mean (±SE) of 3–5 mice per group. Statistical significance, relative to 0% vehicle control (VC), was determined by one-way ANOVA with Dunnett's post-test where *p < 0.05, **p < 0.01. Kruskal-Wallis with Dunn's post-test was conducted for *S100a8* (PFHxA, PFHpA) due to unequal variance.

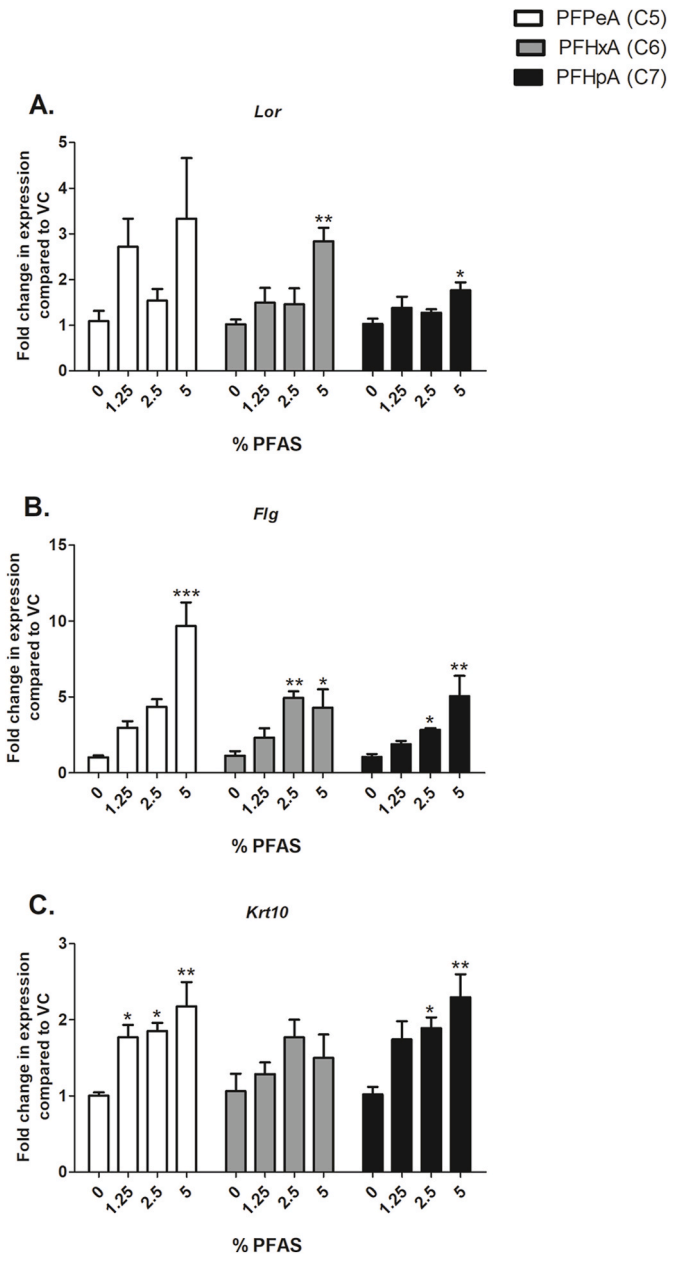

Fig. 6. Skin barrier gene expression following dermal exposure to PFPeA, PFHxA, or PFHpA. Gene expression in the ear following 28 days of PFAS exposure. Changes in *Lor* (A), *Flg* (B), and *Krt10* (C) were evaluated. Data shown as the mean (±SE) of 4–5 mice per group. Statistical significance, relative to 0% vehicle control (VC), was determined by one-way ANOVA with Dunnett's post-test where *p < 0.05, **p < 0.01, ***p < 0.001. Kruskal-Wallis with Dunn's post-test was conducted for *Flg* (PFHpA) due to unequal variance.

(Table 3) (Lau et al., 2007). Consistent with the findings from the studies described in this manuscript, multiple studies with PFAS have shown an increase in liver weights and hepatocellular hypertrophy in rodents following oral and dermal exposure (Chengelis et al., 2009; Han et al., 2020; NTP, 2022; Weatherly et al., 2021).

Further support of liver dysfunction is observed in liver gene expression (Fig. 4). Cd36 contributes to lipid accumulation and metabolic dysfunction and was the only gene that all three alternative PFAS exposures significantly increased to a similar extent (Fig. 4A). Interestingly, the genes involved in fatty acid metabolism (*Pltp, Acox1, Ehhadh*) were most significantly increased with the shortest carbon (C5) PFAS tested, PFPeA (Fig. 4C–E). Pparα gene expression was unchanged in the liver with any PFAS exposure (data not shown). This could be due to expression kinetics, as gene expression analysis was only conducted after the conclusion of the 28-day exposure. This is supported by the upregulation of multiple genes in the liver that are PPARα targets following dermal PFAS exposure (Rakhshandehroo et al., 2010). It is also possible that a PPARα-independent pathway is involved in dermal PFAS exposure. Multiple studies demonstrate activation of PPARγ, PPARβ/δ, constitutive active receptor (CAR), and pregnane X receptor (PXR) in the liver after PFAS exposure (Abe et al., 2017; Bjork et al., 2011; Das et al., 2017; Rosen et al., 2017; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006; Zhang et al., 2017). Pparγ is involved in the development of a fatty liver and steatosis (Inoue et al., 2005; Matsusue et al., 2003) and was significantly increased in the liver with PFPeA and PFHpA exposure (Fig. 4I). These PFPeA-induced alterations in gene expression in the liver correlate with the histology as PFPeA also had the most severe hepatocellular hypertrophy of the three PFAS. Oral PFHxA exposure has also been shown to increase *Acox1* expression (Schrenk et al., 2020) and *Ehhadh* expression (Jiang et al., 2021). Dermal PFBA exposure induced similar findings (Supplemental Table 10). PFOA exposure also increased *Cd36*, *Lpl* (Das et al., 2017), *Acox1* (Abe et al., 2017), *Fabp1*, and *Ehhadh* (Rosen et al., 2009) in the livers of mice. Similar genes were found to be altered with PFAS exposure in human hepatocytes and interestingly short-chain and alterative carboxylic acid PFAS were more potent gene activators than the long-chain legacy carboxylic acid PFOA (Marques et al., 2022). Cd36 contributes to an enlarged fatty liver via transporting lipids to the liver due to metabolic dysfunction. An increase in glucose can be caused by a fatty liver. In addition to PFBA, all three PFAS induced an increase in glucose levels in the current study (Fig. 3B) (Supplemental Table 8) as has oral PFOA exposure (Du et al., 2018; Liu et al., 2018; Zheng et al., 2017). However, dermal PFOA exposure did not elevate glucose in the studies described in this manuscript. Interestingly, PFBA was the only carboxylic PFAS (including both alterative and legacy carboxylic PFAS) that elevated cholesterol (Supplemental Table 8). These data indicate that dermal exposure to the PFAS PFPeA, PFHxA, and PFHpA have similar trends in liver effects compared to oral exposure and legacy PFOA exposure.

The draining LNs were examined to determine whether alternative PFAS dermal exposure altered immune responses. A significant increase in total dLN cellularity was observed for all PFAS tested (Table 1). Interestingly, neither PFOA (Supplemental Table 3) nor PFBA (Supplemental Table 9) induced changes in total dLN cellularity. Further phenotyping revealed that PFAS dermal exposure can induce stimulation of the immune system as evidenced by increases in cell number and frequency of multiple immune subsets supporting a sustained inflammatory response (Table 1). Also, an induction of an adaptive immune response is suggested by an increase in both B-cell number and frequency with PFPeA in the dLN. However, this increase was not observed in the spleen (Supplemental Table 5). The increase in both number and frequency of B-cells was only observed with PFPeA, an increase in number only was seen with PFHpA and no change in B-cells occurred with PFHxA. These trends (increase in frequency and number of B-cells in dLN with no effect on B-cells in the spleen) are similar to what was observed with PFBA (Weatherly et al., 2021). Although no B-cell changes were observed in the dLN with PFOA exposure (Supplemental Table 3), a significant decrease in B-cell number and frequency did occur in the spleen along with a decrease in total cellularity (Supplemental Table 6). While changes in the liver were similar for all compounds in the carboxylate series, only PFOA resulted in decreases in spleen weight and cellularity and thymus weight (Supplemental Fig. 2A), which are signatures for immunotoxicity and immune suppression. This suggests potential different mechanisms of toxicity between alternative and legacy PFAS.

There are many mediators in the skin that may influence immune function and systemic effects. For example, three PPAR isoforms (α, β/δ, and γ) are present in the skin of mice (Michalik and Wahli, 2007) and are involved in lipid metabolism, tissue repair, migration, proliferation, and

differentiation (Michalik et al., 2006). All three isoforms are expressed in T-and B-lymphocytes, DCs, and keratinocytes with expression changing dependent on maturation, activation, etc. (Schmuth et al., 2014) and have anti-inflammatory properties (Furue et al., 2018; Schmuth et al., 2014). A decrease in *Pparα* is associated with skin inflammation (Furue et al., 2018) and this supports our histopathology data where inflammation was observed (Table 4). Correspondingly, we observed decreases in *Pparα* with PFPeA and PFHxA, but not with PFHpA (Fig. 5). Similar to our data, after physical disruption of the skin barrier in human samples *Pparα* expression decreased while *Pparδ* was unchanged (Törmä and Berne, 2009); chemical disruption of the skin barrier decreased Pparα and γ mRNA (Adachi et al., 2013). *Flg* and *Lor* are expressed in the skin and are important in barrier function. While they are usually downregulated in inflammatory skin our data shows an increase in both (Fig. 6) potentially a result of expression kinetics. *Flg* and *Lor* could also be upregulated due to AHR activation as other chemicals are known to activate AHR and enhance *Flg* and *Lor* expression (Sutter et al., 2011). Barrier disruption can also increase expression of the S100's which act as danger signals in the skin (Eckert et al., 2004). *S100a7a* and *S100a8* were upregulated following PFAS exposure in the present study. *In vitro* studies on human keratinocytes and epithelial cells have found that S100a7 exposure increases the mRNA expression of *Flg*, *Lor*, and *Krt10* (Hattori et al., 2014; Nakamura et al., 2021). Additional persistent signs of inflammation after alternative PFAS exposure are evidenced in the skin phenotyping by changes in CD4 T-cells, eosinophils, neutrophils, CD8 T-cells, DCs, and NK cells (Table 2). Pparα and γ activation has been shown to inhibit the number of eosinophils infiltrating to the skin (Hatano et al., 2010). Additionally, the large increases in S100a7 and a8 (Fig. 5C, E) could be due to infiltrating neutrophils in the skin (Ehrchen et al., 2009; Schiopu and Cotoi, 2013). Also, in support of a sustained inflammatory response, is an increase in *Il-6* (PFPeA) and *Tslp* (PFPeA, PFHpA) (Fig. 5). Together these data suggests that alternative PFAS dermal exposure can induce immune-related responses. It is important to note that the immune-related data and the overall toxicity data presented in this manuscript are not completely linear with respect to carbon length and therefore present the possibility of unique mechanisms of toxicity for these compounds potentially influenced by skin absorption and penetration, which has previously been shown for PFOA (Franko et al., 2012; Shane et al., 2020). However, it is important to note that like PFOA, all tested alternative PFAS were shown to be absorbed through the skin.

Skin irritation was observed at the site of application for all three PFAS, mainly with the 5% exposure groups. Because the free acid form of these chemicals was used in the present study, the pH of the PFAS (5%) was measured in PBS, and determined to be 1.49 (PFHpA), 0.96 (PFHxA), and 0.86 (PFPeA). While low pH could be a potential explanation for the skin irritation observed at the site of application, PFOA did not show any signs of irritation at concentrations up to 2% (Shane et al., 2020). Previous work with PFOA suggests that the ionization state can influence skin penetration (Franko et al., 2012). PFAS in an aqueous environment will be highly ionized, which will significantly affect dermal absorption. The amount of moisture in PFAS-containing products will likely play a large role in dermal absorption and this should be considered when evaluating formulations in which these compounds are used, where dermal exposure is possible, such as firefighting foams. Further dermal studies on these compounds in varying buffer environments, including different non-aqueous solvents which are commonly used in firefighting foams, are needed to further our knowledge on alternative PFAS dermal absorption.

It is interesting to note that out of the three PFAS investigated in this study, PFPeA had the least number of epidemiology and animal studies identified but had the most significant changes in gene expression in the liver (Fig. 4) and most severe hepatocellular hypertrophy (Table 3) in the current study. Pérez et al. observed a positive correlation between acidic PFAS having an odd number of carbons (PFPeA, PFHpA) with accumulation in the liver (Pérez et al., 2013). PFPeA is frequently overlooked as serum levels are often below the limit of detection in human samples (Jin et al., 2020; Xu et al., 2020). However, one study found that PFPeA and PFHxA concentrations were 1.5 to 5-fold higher in urine and hair samples than in serum, and PFPeA was the predominant PFAS detected in urine (Kim et al. 2014, 2019). These results are consistent with our murine model as PFOA had the highest concentration in serum (although tested at the lowest concentration) followed by PFHpA > PFHxA > PFPeA (Fig. 1A) supporting a general trend in increased serum levels based on carbon length. The inverseresults occurred in urine, with PFPeA having the highest concentration followed by PFHxA > PFHpA > PFOA (Fig. 1B). In general, it has been determined that alternative PFAS have a shorter half-life than the legacy PFAS and that the alternative PFAS are more quickly excreted (Han et al., 2012), which is supported by our findings. One assumption in the literature is that if a PFAS has a short half-life (with high output in the urine), it has low potential for adverse effects. However, others are challenging this assumption and the current study and previous results with PFBA (Weatherly et al., 2021) help support that this is not the case with certain PFAS.

While studies on alternative PFAS are limited, PFHxA is one of the most studied as it is the main degradation product of fire-fighting foams in current use (Cousins et al., 2016). One critical review determined that PFHxA is not carcinogenic, not a reproductive or developmental toxicant, or an endocrine disruptor (Luz et al., 2019). This was all concluded through oral exposure studies. Dermal toxicity was reviewed in a subsection, however, no experiments with PFHxA dermal exposure were identified. One dermal study using PFHxA ammonium was identified, however, only lack of skin irritation was remarked upon. However, another study using gavage exposure found that PFHxA may disrupt thyroid hormones and decrease epididymal sperm counts (NTP, 2019); and ammonium salt PFHxA may lead to slight developmental effects (Iwai et al., 2019; Loveless et al., 2009). This stresses the data gap with alternative PFAS dermal exposure.

It is notable that the general purpose of this paper is for hazard identification and to confirm dermal absorption of these compounds through analysis of serum and urine. The concentrations used in this manuscript were based on preliminary data generated in our laboratory that showed systemic effects following dermal exposure. There is no data on human serum or urine concentrations after alternative PFAS occupational or consumer product exposure only occurring dermally. Although the serum and urine concentrations in this manuscript are much higher than what has been measured in humans, it should be noted that comparisons between species are difficult as PFAS are excreted at different rates in different species (ATSDR, 2021). Also, human exposures are frequently chronic as opposed to a 28-day exposure with mice and due to the shorter half-life of the alternative PFAS the peak levels in the serum/urine could be being missed in human studies. It is also worth noting that the serum and urine analysis includes only the linear isomer for PFPeA, PFHxA, and PFHpA, however, both linear and branched isomers were measured for PFOA. This could influence the detected concentrations of these PFAS, possibly leading to a lower concentration of the shorter-chain alternative PFAS being reported in the samples.

Alterations in organ weights, histology and phenotyping, serum chemistries, and gene expression along with analytical data, support that these alternative PFAS can be absorbed through the skin. These findings suggest the PPAR isoforms α, δ, and γ are involved in alternative PFAS systemic toxicity and immunological disruption and these effects are possibly due to the disruption of the skin barrier. While similar results to legacy PFAS were demonstrated, unique mechanism of toxicity were also suggested. These results raise additional concerns on both the dermal route of exposure for PFAS and the replacement of legacy PFAS with the alternative counterparts that are being labeled as safer alternatives. Further investigation into PFAS dermal exposure is needed as it is crucial to identify how chemical exposure to the skin influences systemic toxicity.

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CRediT authorship contribution statement

Lisa M. Weatherly: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Hillary L. Shane:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Ewa Lukomska:** Resources. **Rachel Baur:** Writing – review & editing. **Stacey E. Anderson:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. All study data will be made available on the NIOSH Data and Statistics Gateway.

Data availability

All study data will be made available on the NIOSH Data and Statistics Gateway.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113515.

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