Suppression of Humoral Immunity by Perfluorooctanoic Acid is Independent of Elevated Serum Corticosterone Concentration in Mice

Jamie C. DeWitt,*† Carey B. Copeland,† and Robert W. Luebeke†

*Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, North Carolina 27834; and †Immunotoxicology Branch, Experimental Toxicology Division, NHEERL, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27709

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The T-cell–dependent antibody response is suppressed in mice exposed to 3.75, 7.5, 15, and 30 mg PFOA (perfluorooctanoic acid)/kg body weight (bw). Reduced bw accompanied immunosuppression at 15 and 30 mg/kg. We investigated the hypothesis that the observed immunosuppression is secondary to elevated serum corticosterone levels by assessing immune function in adrenalectomized (adx) or sham-operated C57BL/6N female mice exposed to 0, 7.5, or 15 mg PFOA/kg bw in drinking water for 10 days. Bw, primary antibody responses to a T-dependent antigen, clinical serum chemistries related to liver health, and serum corticosterone levels were evaluated. Exposure to 15 mg/kg decreased bw by approximately 10% after 8 days of dosing and until 2 days postdosing in both adx and sham animals; bw of adx animals were still reduced 5 days postdosing. IgM antibody titers were statistically reduced by 15% in sham animals and 18% in adx animals exposed to 15 mg/kg and by 11.8% in adx animals exposed to 7.5 mg/kg. Corticosterone concentrations were elevated by 157% in dosed sham animals relative to control animals and were reduced by 27% in dosed adx animals relative to control animals (neither changes were statistically significant). Clinical serum chemistries related to liver health were not statistically altered by either dose or adrenalectomy. The failure of adrenalectomy to protect mice from the immunosuppressive effects of PFOA indicates that suppression of antibody production is not the result of liver toxicity or stress-related corticosterone production.

Key Words: perfluoronated agents; immunotoxicity; corticosterone.

Perfluoroalkyl acids (PFAAs) are fluorinated compounds used to manufacture myriad consumer products, from adhesives and fire retardant surfaces to nonstick coatings and lubricants. PFAAs undergo chemical, microbial, and photolytic degradation to a limited number of extremely stable degradation products, including perfluorooctanoic acid (PFOA). PFOA is also a polymerization aid used in the manufacture of fluorinated polymers and elastomers. As a result of its use and as a degradation product of other PFAAs, PFOA is widespread in environmental media and has been reported in the serum and tissues of humans and wildlife (Lau et al., 2007). Concerns about PFOA’s toxicological effects prompted a preliminary risk assessment by the U.S. Environmental Protection Agency (EPA) and, although relatively few studies have evaluated PFOA’s immunomodulatory potential, immunotoxicity was cited as an endpoint of concern in a review of the risk assessment by the EPA Science Advisory Board.

The studies cited in the preliminary risk assessment reported reductions in lymphoid organ weights and suppression of de novo antibody synthesis (Yang et al., 2000, 2001, 2002). In these initial studies and in subsequent studies (DeWitt et al., 2008; Loveless et al., 2008), oral exposure to 30 mg PFOA/kg body weight (bw) reduced antigen-specific IgM antibody synthesis in mice. We previously reported that the serum concentration associated with this dose was 1.6 × 10^5 ng/ml after 15 days of exposure. The lowest observed adverse effect level for alteration of primary (IgM) antibody titers was 3.75 mg/kg, with a serum concentration of 7.4 × 10^4 ng/ml, which is approximately 150 times greater than concentrations in highly exposed human populations (DeWitt et al., 2008). In a previous study (unpublished data) the adrenal glands of female C57BL/6 mice exposed to 30 mg/kg PFOA via gavage for 10 or 15 days were enlarged relative to controls. Histopathological analysis revealed increased cytoplasmic vacuolization in the zona fasciculata, the region of the adrenal primarily responsible for glucocorticoid production. Exposure to 30 mg/kg was also associated with reductions in bw and increases in liver weight. Therefore, overt toxicity and stress rather than a direct effect of PFOA on immune responses were considered as potential modulating factors of immune suppression at this dose. In addition, a recently published paper suggested that alterations in lymphoid organs and IgM synthesis in mice exposed to PFOA were the result of severe overt toxicity and stress and that the immune system was not a specific target (Loveless et al., 2008).
To address the potential role of elevated corticosterone production in the suite of effects previously reported (DeWitt et al., 2008), we evaluated lymphoid organ weights, T-cell–dependent antibody responses, clinical serum chemistry parameters related to liver health, and serum corticosterone concentrations in sham-operated or adrenalectomized (adx) C57BL/6 mice exposed to 3.75, 7.5, or 15 mg/kg of PFOA. Our objectives were to determine the influence of corticosterone on PFOA-induced immune suppression and to examine markers of liver toxicity associated with PFOA-induced changes to immune function.

MATERIALS AND METHODS

Animals. C57BL/6N female mice (6–7 weeks of age) were purchased from Charles River Laboratories (Raleigh, NC). Half of the mice were adx, and half received sham adrenalectomy operations (sham); surgeries were performed at Charles River Laboratories prior to arrival at the U.S. EPA. Once at the U.S. EPA’s animal facilities (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), animals were housed in groups of six in polycarbonate cages with hardwood chip bedding (Beta chip; Northeastern Products, Warrensburg, NY). They were provided a 12-h light:dark cycle (light, 0600–1800 h; dark, 1800–0600 h), maintained at 22.3 ± 1.0°C and 50 ± 10% humidity, and given ad libitum access to both food (5000 Prolab RMH 3000; PMI Nutrition International, Richmond, IN) and water. Animals were acclimated for 1 day before dosing began. All procedures employed in this study were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory, U.S. EPA.

Dosing solutions. PFOA was purchased from Fluka Chemical (Steinheim, Switzerland) as its ammonium salt (≥ 98% purity, lot 421207/1 319030). PFOA drinking water dosing solutions were prepared based on total compound weight, as described by DeWitt et al. (2008) at concentrations of 100, 50, or 25 mg/l (to provide doses of 15, 7.5, or 3.75 mg/kg/day, respectively, based on average daily water consumption and animal bw). Dosing water for adx mice was supplemented with 1% sodium chloride to ameliorate sodium deficiency induced by adrenalectomy. Mice received PFOA-containing drinking water for 10 consecutive days. Dosing water was changed and water consumption per cage (based on water bottle weights) was recorded twice weekly. Vehicle controls received deionized water (sham) or deionized water supplemented with 1% sodium chloride (adx) for 10 days.

Experimental design. Animals arrived in two separate groups; one group was used for clinical chemistry analysis of serum and the other group was used for measurement of corticosterone and antigen-specific IgM antibody titers. Animals used for clinical serum chemistries were not immunized. Each endpoint (clinical serum chemistry, corticosterone, IgM) group contained five to seven animals. Bw and Water Consumption

RESULTS

Bw and Water Consumption

Only bw of animals used for measurement of IgM antibody titers are presented. Bw of sham animals (Fig. 1A) dosed with 15 mg/kg PFOA were 7.6% lower than bw of control animals at the eighth day of dosing and 9.8% lower 2 days postdosing ($p < 0.05$). Bw of adx animals (Fig. 1B) dosed with 15 mg/kg...
PFOA were 9.6% lower, on average, from the eighth day of dosing until 5 days postdosing, compared with control animals ($p < 0.05$). In addition, bw of adx animals dosed with 7.5 mg/kg PFOA were 10.3% lower than bw of control animals two days postdosing ($p < 0.05$). Water consumption did not vary by dose (data not shown).

**Clinical Serum Chemistries**

Triglyceride level was the only clinical serum chemistry parameter that demonstrated a statistically significant dose-dependency (Tables 1 and 2). Triglyceride levels in sham animals dosed with 3.75, 7.5, or 15 mg/kg PFOA for 5 days were 44.8, 57.8, and 61.2% lower, respectively, than levels measured in sham control animals. In adx animals only the 7.5 and 15 mg/kg groups were statistically lower than the adx control animals (59.9 and 75.2%, respectively). On average, triglyceride levels were lower after ten days of exposure compared with five days of exposure, but the differences between triglyceride levels after five or ten days of exposure were not statistically significant. A few other clinical chemistry parameters were statistically elevated or decreased relative to control responses, but no changes indicated a trend associated with dose, duration of exposure, or adrenalectomy (Tables 1 and 2). In general, clinical serum chemistry parameters of dosed animals did not differ statistically from those of control animals.

**Corticosterone and IgM Antibody Titers**

Baseline corticosterone levels in serum collected prior to dosing were $125.6 \pm 59.0$ ng/ml for the sham animals and 5.7 ± 0 ng/ml for the adx animals (all adx animals were below LOD). After 10 days of PFOA dosing, average corticosterone

![Graph A: Mean Body Weight (g) vs. Days of Dosing](imageA)

![Graph B: Mean Body Weight (g) vs. Days of Dosing](imageB)

**FIG. 1.** Bw of (A) sham-operated or (B) adx female C57BL/6N mice exposed to various doses of PFOA for 10 days and used for measurement of IgM antibody titers (mean ± SEM; $N = 6$/dose). PD, postdosing. *Statistically different from 0 mg/kg group ($p < 0.05$).

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>0 mg/kg</th>
<th>3.75 mg/kg</th>
<th>7.5 mg/kg</th>
<th>15 mg/kg</th>
</tr>
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<tbody>
<tr>
<td>ALP (U/I)</td>
<td>184.92±11.45</td>
<td>154.45±7.12</td>
<td>139.88±9.61</td>
<td>147.00±8.08</td>
</tr>
<tr>
<td>ALT (U/I)</td>
<td>39.52±2.50</td>
<td>26.96±1.78</td>
<td>43.88±0.93</td>
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<td>AST (U/I)</td>
<td>121.56±17.86</td>
<td>73.53±4.70</td>
<td>104.07±10.24</td>
<td>76.58±3.38</td>
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<tr>
<td>SDH (U/I)</td>
<td>46.43±1.03</td>
<td>33.05±1.58</td>
<td>39.31±3.32</td>
<td>37.95±2.35</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>26.36±1.53</td>
<td>22.21±2.02</td>
<td>22.27±2.69</td>
<td>19.63±0.98</td>
</tr>
<tr>
<td>CREA (mg/dl)</td>
<td>0.42±0.01</td>
<td>0.35±0.02</td>
<td>0.46±0.03</td>
<td>0.47±0.02*</td>
</tr>
<tr>
<td>GGT (U/I)</td>
<td>8.59±0.40</td>
<td>8.01±0.34</td>
<td>8.24±0.70</td>
<td>9.43±0.72</td>
</tr>
<tr>
<td>GLUC (mg/dl)</td>
<td>190.86±7.95</td>
<td>190.75±9.03</td>
<td>189.63±2.47</td>
<td>210.09±9.46</td>
</tr>
<tr>
<td>CHOL (mg/dl)</td>
<td>109.72±4.79</td>
<td>85.07±5.22</td>
<td>110.96±4.02</td>
<td>77.12±3.57</td>
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<tr>
<td>TRIG (mg/dl)</td>
<td>124.87±11.48</td>
<td>132.78±20.66</td>
<td>68.99±5.21</td>
<td>80.00±19.71</td>
</tr>
<tr>
<td>PROT (g/dl)</td>
<td>5.94±0.11</td>
<td>5.18±0.23</td>
<td>5.65±0.13</td>
<td>5.19±0.13</td>
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<tr>
<td>LDH (U/I)</td>
<td>320.57±39.84</td>
<td>176.50±19.32</td>
<td>293.92±68.65</td>
<td>222.69±19.18</td>
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</tbody>
</table>

*Note.* Mean ± SEM. *Statistically different from corresponding (sham or adx) control group ($p < 0.05$).
concentrations in sham animals were approximately 157% greater in dosed animals compared with control animals (Fig. 2A; only the 15 mg/kg group was statistically different from controls). Five days after dosing ended, average corticosterone levels in sham animals were 73% greater in dosed animals compared with control animals (Fig. 2A; no groups statistically different from controls). Average corticosterone concentrations in adx animals were 27% lower in dosed animals compared with control animals after 10 days of PFOA dosing (Fig. 2B; only the 15 mg/kg group was statistically different from controls) and within 10% of each other 5 days after dosing ended (Fig. 2B).

Dosing with 15 mg/kg PFOA for 10 days reduced SRBC-specific IgM antibody titers (Fig. 3) by 18% relative to controls in sham animals and by 15.4% in adx animals (p < 0.05). Dosing with 7.5 mg/kg PFOA also reduced IgM antibody titers by 11.8% relative to controls in adx animals (p < 0.05).

### DISCUSSION

The data presented here indicate that suppression of T-cell–dependent antibody responses (TDAR) in PFOA-treated mice is independent of increased serum corticosterone levels and liver toxicity as measured by clinical serum chemistries. In this study, we evaluated clinical serum chemistry parameters related to liver health, serum corticosterone levels, and TDAR in sham-operated or adx adult C57BL/6N female mice dosed with one of several concentrations of PFOA for ten days to verify corticosterone levels or changes to clinical serum chemistries as factors in suppression of IgM TDAR. None of the clinical serum chemistry parameters measured indicated a gross alteration in liver health of animals in the study. Dosing with 15 mg/kg of PFOA statistically reduced IgM antibody titers in both sham and adx animals and 7.5 mg/kg PFOA also

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**TABLE 2**

Summary of Clinical Blood Chemistry Parameters in Mice Treated with PFOA for 10 Days (N = 6/group; Sera Collected 1 day after Dosing Ended)

<table>
<thead>
<tr>
<th></th>
<th>0 mg/kg</th>
<th>3.75 mg/kg</th>
<th>7.5 mg/kg</th>
<th>15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/l)</td>
<td>217.12±42.02&lt;sub&gt;a&lt;/sub&gt;</td>
<td>202.62±26.53</td>
<td>247.82±97.68</td>
<td>139.15±22.06</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>51.51±14.62</td>
<td>128.22±24.80</td>
<td>79.26±33.87</td>
<td>282.23±193.54</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>92.30±6.33</td>
<td>106.00±8.86</td>
<td>123.73±15.20</td>
<td>217.10±3.48</td>
</tr>
<tr>
<td>SDH (U/l)</td>
<td>54.60±16.72</td>
<td>61.88±8.87</td>
<td>45.50±10.15</td>
<td>68.78±24.88</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>23.67±2.02</td>
<td>19.97±1.08</td>
<td>25.12±2.14</td>
<td>23.08±3.25</td>
</tr>
<tr>
<td>CREA (mg/dl)</td>
<td>0.47±0.03</td>
<td>0.44±0.03</td>
<td>0.43±0.02</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>GGT (U/l)</td>
<td>7.63±0.43</td>
<td>8.24±0.48</td>
<td>7.60±0.43</td>
<td>9.23±0.36</td>
</tr>
<tr>
<td>GLUC (mg/dl)</td>
<td>212.03±6.75</td>
<td>190.08±11.84</td>
<td>182.62±8.55</td>
<td>184.96±12.49</td>
</tr>
<tr>
<td>CHOL (mg/dl)</td>
<td>82.05±5.58</td>
<td>99.30±3.08</td>
<td>86.44±5.05</td>
<td>83.98±6.23</td>
</tr>
<tr>
<td>TRIG (md/dl)</td>
<td>81.59±15.87</td>
<td>66.19±7.72</td>
<td>63.02±9.40</td>
<td>57.75±14.61</td>
</tr>
<tr>
<td>PROT (g/dl)</td>
<td>5.66±0.16</td>
<td>6.08±0.16</td>
<td>5.85±0.16</td>
<td>5.46±0.20</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>333.48±86.86</td>
<td>239.96±30.23</td>
<td>404.14±59.89</td>
<td>379.61±80.67</td>
</tr>
</tbody>
</table>

Note. *Mean ± bSEM. *Statistically different from corresponding (sham or adx) control group (p < 0.05).

**FIG. 2.** Serum corticosterone (ng/ml) of (A) sham-operated or (B) adx female C57BL/6N mice exposed to various doses of PFOA for 10 days (mean ± SEM; N = 6/dose). Serum was collected one day prior to dosing (baseline), after 10 days of dosing, or at the time collection of serum for IgM analysis (5 days of recovery). *Statistically different from time-matched 0 mg/kg group (p < 0.05).
reduced titers in adx animals. In addition, serum corticosterone levels were elevated in dosed animals relative to control animals in the sham group. As expected, relatively low levels of corticosterone were measured in the adx animals (16.0–51.9 ng/ml range).

Yang et al. (2002) evaluated the effects of PFOA on antigen-specific antibody responses and lymphocyte proliferation in response to T- or B-cell mitogens in male C57BL/6 mice fed a diet of 30 mg PFOA/kg for 15 days. Antigen-specific IgM and IgG serum titers were suppressed relative to responses of unexposed animals. In a set of studies designed to corroborate the immune alterations reported by Yang et al. (2002), we previously reported (DeWitt et al., 2008) suppression of IgM antibody titers in female C57BL/6 given 3.75, 7.5, 15, and 30 mg/kg of PFOA in drinking water. At the two lower doses, no effects on lymphoid organ weights or bw were observed. However, at the 15 and 30 mg/kg doses, bw and lymphoid organ weights were reduced, and at the 30 mg/kg dose (in a previous study, unpublished), cytoplasmic vacuolization in the zona fasciculata of the adrenal was increased, suggesting the potential for stress-induced immune suppression from increased corticosterone production. In addition, Loveless et al. (2008) reported suppression of IgM antibody titers in male CD-1 mice exposed to 10 or 30 mg/kg PFOA for 28 days, confirming our results and the results of Yang et al. (2002) in another strain of mice. However, Loveless et al. (2008) asserted that these immune findings represented secondary responses to systemic toxicity and stress as indicated by bw loss and elevated serum corticosterone levels.

Corticosterone is a glucocorticoid that increases in response to many stressors. Elevated production of corticosterone is immunosuppressive, although duration of exposure to elevated levels is critical in determining the outcome. The relationship between increased corticosterone concentration and changes in immune function has been represented by the area under the curve (AUC) for serum corticosterone (Pruett, 2001, 2003; Pruett and Fan, 2001; Pruett et al., 1999, 2003, 2007). The cumulative effects of hormones on downstream functions are often evaluated by calculating the AUC, which can be thought of as total hormonal output over a given time period (Pruessner et al., 2003). The AUC concept for corticosterone illustrates the linear relationship between the duration of corticosterone elevation and suppression of certain immune parameters. Circulating leukocytes, lymphoid organ weights, and changes to lymphoid organ histopathology occur at AUCs between 1380 and 4188 ng/ml/h (Pruett and Hébert, 2007). However, antibody production, the response that we evaluated, is relatively insensitive to increases in corticosterone; Pruett and Fan (2001) reported that SRBC-specific IgG antibodies in mice were only suppressed at AUCs greater than 3000 ng/ml/h, and enhanced at lower AUCs.

In the current study corticosterone was only evaluated at three time points separated by days, rather than hours, so we were not able to calculate the AUC for corticosterone. However, we did measure serum corticosterone immediately after animals had experienced handling (restrained), injection (immunized with SRBCs), and chemical exposure (dosed with PFOA for 10 days). The highest corticosterone concentration that we measured in any mouse was 380 ng/ml. In comparison, in a study of the effects of atrazine and ethanol on stress responses in mice, peak corticosterone concentrations were 1200 and 800 ng/ml, respectively (Pruett et al., 2003), which were two to four times the peak corticosterone levels measured in our study. Pruett and Fan (2001) reported that an AUC greater than 3000 ng/ml/h was necessary to reduce IgG antibody titers and Neigh et al. (2004) reported that an AUC of approximately 250 ng/ml/h was necessary to reduce IgM antibody titers. Given that the average corticosterone concentrations for all of our groups were less than 200 ng/ml, it is unlikely that we achieved an AUC sufficient to reduce IgM antibody titers in our study.

Serum corticosterone levels associated with suppression of IgM antibody titers in CD-1 mice exposed to 30 mg/kg PFOA for 28 days were between 400 and 500 ng/ml, which were more than 200% greater than levels in control animals (Loveless et al., 2008). Fang et al. (2008) reported increases in cortisol, changes in lymphocyte populations, and alteration in cytokines in BALB/c mice exposed to 3 or 5 mg/kg perfluoronananoic acid via gavage for 14 days. Increases in cortisol in exposed animals were approximately 50% greater relative to control animals, but concentrations were all lower than 100 ng/ml. In both of these studies, the authors suggested that the immunomodulatory effects observed were from activation of the HPA axis by the chemical under study. Loveless et al. (2008) suggested that the immune suppression was the result of a stress response and Fang et al. (2008) suggested that the observed changes in immune parameters were associated with...
increases in cortisol levels. Our data contradict these assertions and indicate that immunosuppression is independent of elevated corticosterone.

PFOA ligates the peroxisome proliferator–activated receptor alpha (PPAR-α), a ligand-activated transcription factor that regulates gene expression related to lipid and glucose homeostasis, cell proliferation and differentiation, and inflammation. PPAR-α agonists, including PFOA, have been reported to reduce inflammation induced by carageenan (Taylor et al., 2002, 2005). When coadministered with RU-486, a glucocorticoid receptor (GR) partial agonist, the anti-inflammatory effect of PFOA was not altered, indicating that GR receptors do not mediate the effects of PFOA (Taylor et al., 2005). Taylor et al. (2005) therefore suggested that a mechanism mediated by endogenous corticosterone release following activation of the HPA axis was not sufficient to explain PFOA’s anti-inflammatory effects.

Although the involvement of the HPA axis at higher doses of fluorinated compounds is possible, it is not sufficient to explain immune suppression at lower doses or at doses where signs of generalized toxicity (i.e., loss in bw or decreased bw gain) are not evident. Although we do not discount that overt toxicity is likely at higher doses (i.e., 15 and 30 mg/kg PFOA), where loss of bw, even when adjusted for increases in liver weight, also occurred, a stress response is insufficient to explain immune suppression at 7.5 mg/kg PFOA in the adx animals. The lack of dose-dependent changes to clinical serum chemistries also indicates that liver toxicity, as a symptom of overt toxicity, was not induced by PFOA exposure or adrenalectomy at lower doses. As glucocorticoids autoregulate GR expression, we are evaluating GR levels in livers of mice exposed to PFOA. Changes in GR levels would be an indicator of PFOA-induced increases in corticosterone during the exposure period. However, changes in GR levels would not explain the reductions in antibody synthesis we observed in the adx animals.

In our previous study, female C57BL/6N mice exposed to 3.75 or 7.5 mg/kg PFOA for 15 days had suppressed IgM antibody titers but no changes in body, spleen, or thymus weights (DeWitt et al., 2008). In the current study, suppression of IgM antibody titers was observed in both sham and adx animals exposed to PFOA for 10 days. Although these doses were associated with about 10% weight loss in the adx animals relative to controls (even after bw was adjusted for increases in liver weights), the absence of significant changes to clinical serum chemistries and low levels of corticosterone indicate that our immune findings do not represent secondary responses to systemic toxicity and stress. Peden-Adams et al. (2008) observed suppression of both T-cell–dependent (SRBC-specific) and T-cell–independent (trinitrophenyl-specific) antibody synthesis in mice exposed to perfluorooctane sulfonate for 28 days. No changes in bw, lymphoid organ weights, or lymphoid cell populations were observed at the lower doses associated with immune suppression. Although they did not measure corticosterone, Peden-Adams et al. (2008) hypothesized that alterations in B cells or antigen presenting cells were associated with the reduced antibody responses. We are also evaluating PFOA’s ability to affect responses to T-cell–independent antigens. In addition, we are consulting with the epidemiologists studying PFOA-exposed humans in West Virginia to determine if titers to immunizations have been affected by PFOA. Results of such studies will help to direct future immunotoxicity studies with PFOA and other PPAR-α ligands. As human PPAR-α expression is only one-tenth that of rodents (Kennedy et al., 2004), alterations to antibody synthesis in humans may suggest that a PPAR-α–independent mechanism influences PFSA immunotoxicity.

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