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Age- and Species-Dependent Infiltration of Macrophages into the Testis of Rats and Mice Exposed to Mono-(2-Ethylhexyl) Phthalate (MEHP)[1]

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ABSTRACT

The mechanism by which noninfectious testicular inflammation results in infertility is poorly understood. Here the infiltration of CD11b+ immunoreactive testicular interstitial cells (neutrophil, macrophages, dendritic cells) in immature (Postnatal Day [PND] 21, 28, and 35) and adult (PND 56) Fischer rats is described at 12, 24, and 48 h after an oral dose of 1 g/kg mono-(2-ethylhexyl) phthalate (MEHP), a well-described Sertoli cell toxicant. Increases of CD11b+ cells are evident 12 h after MEHP exposure in PND 21 and 28 rats. In PND 28 rats, CD11b+ cells remained significantly elevated at 48 h, while in PND 21 rats, it returned to control levels by 24 h. The peak number of CD11b+ cells in PND 35 rat testis is delayed until 24 h, but remains significantly elevated at 48 h. In PND 56 rats, no increase in CD11b+ cells occurs after MEHP exposure. In PND 21, 28, and 35 rats, a significant increase in monocyte chemoattractant protein-1 (MCP-1) by peritubular myoid cells occurs 12 h after MEHP. Interestingly, MEHP treatment of C57BL/6J mice did not incite an infiltration of CD11b+ cells at either PND 21 or 28. The peak level of germ cell apoptosis observed 24 h after MEHP exposure in young rats is not seen in mice at any age or in PND 56 rats. Taken together, these findings implicate MCP-1 released by peritubular myoid cells in provoking the migration of CD11b+ cells into the immature rat testis early after MEHP exposure and point to a role for CD11b+ cells in triggering germ cell apoptosis in an age- and species-dependent manner.

interstitial cells, macrophage, myoid cells, reproductive immunology, toxicology

INTRODUCTION

Phthalic acid esters (phthalates) are a class of compounds that are incorporated into cosmetics, food packaging, biomedical devices, and in polyvinyl chloride and plastic products. These agents are not covalently bound to the final product and as a result are found widely distributed in the environment, human tissues, and urine [1–3]. Di(2-ethylhexyl) phthalate (DEHP) is the most abundantly produced phthalate. Upon consumption, DEHP is rapidly hydrolyzed in the gastrointestinal tract to its primary active metabolite mono-(2-ethylhexyl) phthalate (MEHP) [4]. Multiple studies demonstrate that urinary levels of phthalate metabolites are higher in children than adults (reviewed in [5]).

MEHP is a well-described Sertoli cells toxicant [6–10]. The mechanisms by which phthalates exert their toxic effects vary between different developmental ages [11]. In peripubertal animals, phthalate-induced injury to Sertoli cells leads to a disruption of the Sertoli cell cytoskeleton and tight junctions followed by increased expression of FasL on Sertoli cell membrane, triggering apoptosis and/or detachment of Fas-expressing germ cells [12]. Peripubertal rodents are particularly sensitive to phthalate-induced testicular injury compared to adult rodents [7]. The effects of MEHP are species-dependent with a higher rate of germ cell apoptosis induced in rats compared to mice [7, 13]. During the peripubertal period, the initial first wave of spermatogenesis is nearing completion, a key process at this time in development for the establishment of sexual maturation and testicular competence. The first wave is characterized by an increase in germ cell apoptosis in which Sertoli cells limit the germ cell population to numbers that they can support [13, 14]. This is a particularly sensitive time of testicular development because at this time the length and diameter of seminiferous tubules increase and the blood testis barrier (BTB) and immune privilege of the testis becomes fully established and activated [6, 15].

Exposures to phthalates are implicated in a variety of immune disorders. These include allergies, asthma, dermatitis, and inflammation [16, 17]. Phthalates induce the production of inflammatory mediators from lung cells in vitro and exert adjuvantlike effects on immune responses within the respiratory system [16, 18]. Within the testis, immune cell infiltration has been observed through histological/morphological examination in response to MEHP exposure to peripubertal rats [19, 20]. However, the type of immune cells and the chemokine inducing infiltration have not been characterized.

The testis is an immune-privileged organ that protects autoantigenic haploid germ cells from assault by the immune system. The immune privilege of the testis is primarily attributed to the nonmyeloid Sertoli cells. Sertoli cells share several features with monocytes/macrophages, including phagocytic activity, production of inflammatory mediators, and expression of Toll-like receptors [21, 22]. Under normal conditions, leukocytes are present within the testicular interstitium, including T cells, natural killer cells, mast cells, eosinophils, and testicular macrophages (TMs) [15]. Of these, the TMs are the largest population, consisting of anti-inflammatory resident TMs (CD68−CD163+) and newly arrived monocytelike TMs (CD68+CD163−) [23].

The testis can become inflamed due to multiple mechanisms resulting in the activation of innate and adaptive immune cells [15, 22]. Inflammation occurs when specific pathogenic molecules (endotoxins), phagocytosis of opsonized particles, and/or immune complexes activate the resident TMs or Sertoli cells. Testicular inflammation can be induced through

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noninfectious origins as well [24]; however, the mechanisms are poorly understood.

Utilizing the established model of MEHP-induced Sertoli cell injury, a paracrine feedback loop between Sertoli cells and germ cells has been revealed that initiates germ cells to undergo apoptosis via FasL-Fas signaling [12, 25–27]. Central to this pathway is the activation of matrix metalloproteinase 2 (MMP2) that leads to the production of soluble tumor necrosis factor-α (sTNF-α) and a disruption of the specialized BTB junctions between Sertoli cells [26, 28]. Because sTNF-α is a widely recognized immune modulatory protein, a logical extension of this work is to assess the participation of immune cells in the well-described model of MEHP-induced testicular injury. The present study reveals a species-, age-, and dose-dependent response of macrophage infiltration into the testis as a result of MEHP-induced testicular injury and the possible participation in the induction of germ cell apoptosis, thus providing insight into the effects of immune compromise at a critical stage of development.

MATERIALS AND METHODS

Animals and MEHP Treatment

Male Fischer rats were purchased from Charles River. Breeding pairs of C57BL/6J mice were purchased from The Jackson Laboratory. Animals were maintained in a controlled temperature (22°C ± 0.5°C) and lighting (12L:12D) environment and allowed to acclimate for 1 wk before experimental challenge. Standard laboratory chow and water were supplied ad libitum. All the animal procedures were performed in accordance with the guidelines and approval of the University of Texas at Austin's Institutional Animal Care and Use Committee.

Groups of exact age Postnatal Day (PND) 21, 28, and 35 and approximate age PND 56 (± 3 days) male Fischer F344 rats and PND 21 and 28 male mice were treated with a single oral dose of MEHP (1 g/kg in corn oil, 97.3% purity; Wako Chemicals) or equivalent volume of vehicle (corn oil, 2 ml/kg). These ages were selected to encompass during (PND 21 and 28) and after (PND 35 and 56) the establishment and activation of the Sertoli cell barrier [15]. This is an established dosing regimen for MEHP that results in a well-characterized Sertoli cell injury at these particularly sensitive developmental ages [8]. Dose-response (1, 0.75, 0.5, or 0 g/kg MEHP in corn oil) studies were performed on the most sensitive rats, that is, PND 28 rats. The metabolite, MEHP, was used to ensure that the differences between age and species were not due to the metabolism of the parent compound, DEHP [7]. At 12, 24, and 48 h after the treatment, animals (at least 3 animals/time point/treatment) were anesthetized using a ketamine (100 mg/ml) and xylazine (20 mg/ml) cocktail (0.1 mg/10 g body weight; Animal Health International) and perfused with phosphate buffered saline (PBS) and heparin (10 units/ml) until complete exsanguination. The testes of rats were removed, weighed, and one testis was either snap frozen in liquid nitrogen and stored at −80°C (for immunostaining and enzyme-linked immunosorbent assay [ELISA]) or fixed in Bouin fixative (PND 28 rat for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling [TUNEL] assay). The other testis was placed in PBS (3 ml) on ice until interstitial cell collection. For mice, both testes were placed in PBS (3 ml) on ice and used for interstitial cell collection. Additional mice were treated as described above and testis were collected with one testis snap frozen in liquid nitrogen and stored at −80°C and the other fixed in Bouin fixative (PND 21 mouse for TUNEL assay).

Interstitial Cell Collection and Flow Cytometry

Single-cell suspensions from the interstitial space of rat or mouse testis were reacted with a panel of antibodies to identify leukocyte subpopulations. Interstitial cells were obtained by decapsulating one (rat) or two (mouse) testes per rodent ($n \geq 3$/treatment/time point) in 3 ml of ice-cold PBS. Both testes from mice were collected due to the lower number of interstitial cells/testis. After the tunica was removed, 1 ml of Dulbecco-modified Eagle medium plus 1% collagenase was added, and seminiferous tubules were gently teased apart using fine forceps. Seminiferous tubules were then rinsed with 1 ml of PBS. Interstitial cells were centrifuged (5 min, 1200 rpm, 4°C Beckman table top), filtered through 70 μm cell strainer, resuspended in 1 ml of PBS, and adjusted to approximately 1×10^6 cells/ml. Cells were centrifuged (1200 rpm at 4°C) for 5 min, resuspended in 200 μl of FACs buffer (PBS plus 2% serum) plus CD4-PE (1:200; Life Technologies), CD8-FITC (1:200; Life Technologies), and CD11b-APC (1:200; eBiosinces). Cells were incubated on ice in the dark for 30 min, then washed with 3 ml of FACs buffer, centrifuged at 1200 rpm for 5 min at 4°C, and resuspended in 0.5 ml FACs buffer containing propidium iodide (2 μg/ml). For each sample, 50 000 cells from the testis were analyzed on the BD LRSFortesa flow cytometer with FACSDiva software and interpreted with FlowJo software. Single-cell suspensions from lymph nodes were reacted without and with single or mixes of antibodies to set the gates and compensation, and to eliminate autofluorescence. Only live cells were used for the analysis. The total number of CD11b+ cells (% CD11b+ × number of interstitial cells) was determined for each rodent. The fold-change was calculated for each rodent as the number of CD11b+ cells for MEHP exposure/CD11b+ cells (average) control for each age, dose, and time point.

Immunohistochemistry

Cross-sections (6 μm) of frozen rat testes embedded in optimal cutting temperature compound (Tissue-Tek) were mounted on Superfrost Plus glass slides and air-dried. Frozen sections were washed in PBS twice for 3 min each, then fixed in acetone at −20°C for 5 min. Sections were incubated with 3% H_2O_2 to block endogenous peroxidase activity and then with blocking buffer (10% horse serum). Sections were incubated with mouse anti-CD68 (1:100; BD Pharmingen) for 1 h at room temperature. Negative controls were run simultaneously and were incubated with blocking serum only instead of primary antibody. Immunodetection was performed by standard procedure using VectaStain ABC kit (Vector Labs) and 3,3′-diaminobenzidine substrate (Vector Labs). At least two cross-sections from three rodents/time point/age/treatment were analyzed.

TUNEL Assay

The presence of apoptotic fragmentation of DNA in paraffin-embedded or frozen testis cross-sections was determined by TUNEL analysis using the ApopTag kit (EMD Millipore). According to previous studies [26, 29], the apoptotic index (AI) was calculated as the percentage of essentially round seminiferous tubules containing more than three TUNEL-positive germ cells in each cross-section. For each rodent, at least two cross-sections and at least 200 seminiferous tubules were analyzed. To compensate for smaller seminiferous tubules in younger animals, at least 400 tubules were counted in PND 21 rodents.

ELISA for Monocyte Chemoattractant Protein-1

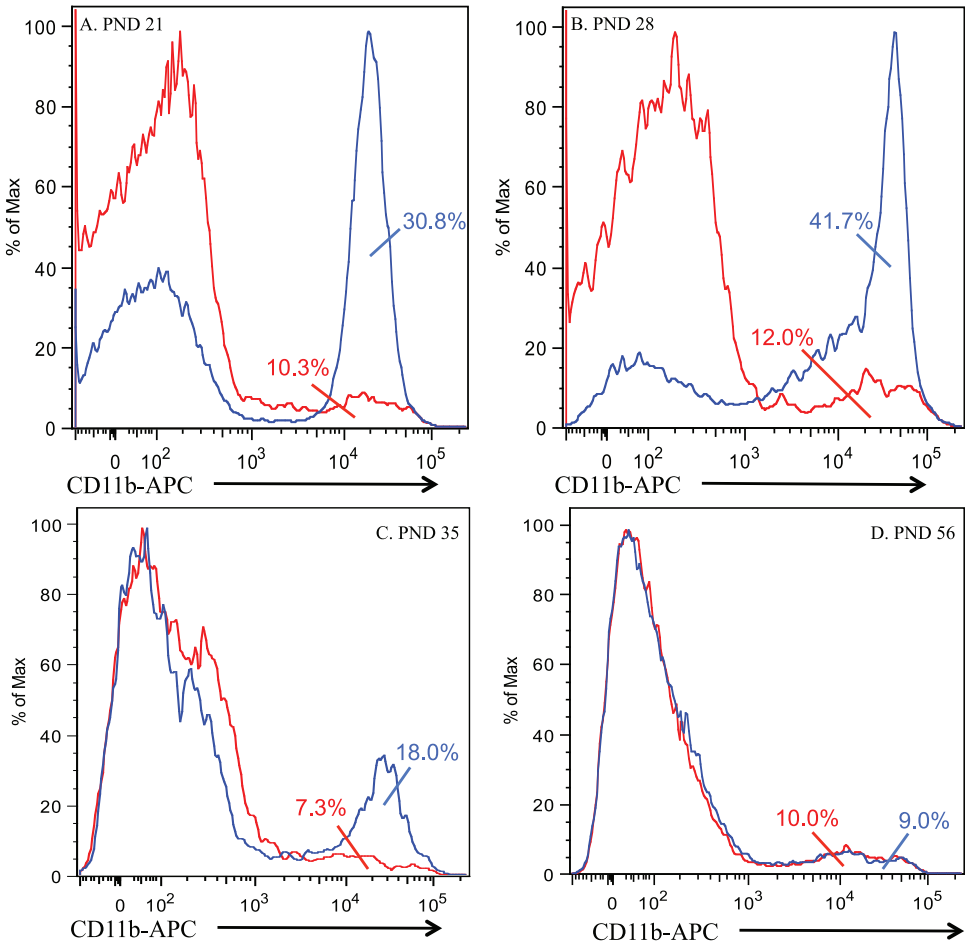
The level of testicular monocyte chemoattractant protein-1 (MCP-1) was quantified using a commercially available MCP-1 rat ELISA kit for tissue extracts (Abcam). Total protein from rat testis tissue was prepared as published previously [28]. Briefly, snap frozen testes were thawed on ice, homogenized in radioimmunoprecipitation assay buffer plus proteinase inhibitors, and total protein content was determined using Lowry protein assay. A total of 25 μg of protein/rat testis was assayed ($n = 3$/treatment/time point/age).

Immunofluorescence

Cross-sections (6 μm) of frozen rat testes embedded in optimal cutting temperature compound (Tissue-Tek) were mounted on Superfrost Plus glass slides and air-dried. Frozen sections were washed in PBS twice for 3 min each, then fixed in acetone at −20°C for 5 min. Sections were incubated with 3% H_2O_2 to block endogenous peroxidase activity and then incubated in blocking buffer (10% goat serum). Sections were incubated with rabbit anti-MCP-1 (1:500; Thermoscientific) for 1 h at room temperature, then incubated in Alexa Fluor 488-conjugated anti-rabbit antibody (1:500; Life Technologies) for 1 h and mounted with Vectashield Mounting Medium (Vector Labs). Negative control sections were run simultaneously in which primary antibody was omitted and only secondary antibody was applied. At least two cross-sections from three rodents/time point/age/treatment were analyzed. Fluorescent signals were detected using excitation/emission wavelengths of 495 nm/519 nm, respectively. All the sections were imaged using Nikon Eclipse microscope and captured with Nikon Cool-SNAP digital camera. Images were processed and analyzed using NIS Elements software.

Statistical Analysis

All the experimental groups were performed in at least triplicate. The data were subjected to Student *t*-test or a parametric one-way analysis of variance (ANOVA) followed by Tukey test for post hoc comparisons. Statistical significance was considered to be achieved when $P < 0.05$ unless otherwise stated.



E

Fold-Change in CD11b+ Cells After MEHP

	PND 21	PND 28	PND 35	PND 56
12 h	9.3±3.7*	12.4±6.3*	2.0±1.0	1.0±0.1
24 h	1.5±0.6	9.6±4.6*	2.8±1.3*	1.2±0.4
48 h	1.0±0.1	3.4±1.0*	1.6±0.2*	1.2±0.2

FIG. 1. Age-dependent MEHP-induced testicular infiltration of CD11b+ cells in rats. Expression of CD11b+ cells in single cell suspension of live testicular interstitial cells after MEHP treatment of PND 21 (**A**), 28 (**B**), 35 (**C**), and 56 (**D**) rats after 12 h of MEHP exposure. Blue represent MEHP-treated rats (1 g/kg, p.o.) and red is vehicle-treated rats (corn oil, equivalent volume). The fold-change increases in CD11b+ cells after MEHP treatment (1 g/kg, p.o.) at each age and time point are summarized in the table (**E**). Asterisks (*) indicate significant differences between treatments at specified time points ($P < 0.10$, Tukey honestly significant difference [HSD] test; PND 21: n = 4, PND 28: n = 6, PND 35: n = 6, PND 56: n = 3 per time point/treatment).

RESULTS

Age-Dependent MEHP-Induced Infiltration of CD11b+ Cells in Rats

In Fischer rats, A single oral dose of MEHP (1 g/kg, per os [p.o.]) induced testicular inflammation in a time- (12, 24, and 48 h) and age-dependent manner (PND 21, 28, 35, and 56). Preparations of isolated testicular interstitial cells were probed with antibodies against CD11b (neutrophil, macrophages, and dendritic cells), CD4 (T helper cells), and CD8 (cytotoxic T cells) and were quantified by flow cytometry. In PND 28 rats, the number of CD11b+ cells significantly increased at 12 h (12.4-fold increase) after exposure to MEHP and remained significantly elevated at 24 and 48 h compared to controls (9.6- and 3.4-fold increases, respectively; Fig. 1, B and E). In PND 21 rats, the total number of CD11b+ cells were also significantly increased after 12 h (9.3-fold increase; Fig. 1A) but quickly returned to control levels by 24 and 48 h (1.5- and 1-fold increases, respectively). The peak infiltration of CD11b+ cells for PND 35 rats was delayed until 24 h (2.8-fold increase) and still remained significantly elevated at 48 h (1.6-fold increase) compared to controls. The increase in PND 35 rats was not as robust as in the PND 21 and 28 rats. In adult rats (~PND 56), there was no observed increase in infiltration

of CD11b+ cells in response to MEHP at all the time points collected (Fig. 1, D and E).

At all ages and time points collected, there were no differences between treatments in the number of CD4+ or CD8+ T cells (data not shown). With increasing age, there was a direct correlation to the number of cells collected from the testis of all the treatment groups. MEHP-treated rats within each age group had a greater number of total interstitial cells collected; however, this did not reach significance.

Dose-Response of MEHP-Induced Infiltration of CD11b+ Cells in PND 28 Rats

A single oral dose of MEHP (1, 0.75, or 0.5 g/kg, p.o.) induced testicular inflammation in a time- (12, 24, and 48 h) and dose-dependent manner in PND 28 Fischer rats. Preparations of isolated testicular interstitial cells were probed with antibodies against CD11b (neutrophil, macrophages, and dendritic cells), CD4 (T helper cells), and CD8 (cytotoxic T cells) and were quantified by flow cytometry. As observed above, in 1.0 g/kg MEHP-treated rats at all the time points, there was a significant increase in the number of CD11b+ cells (17.1-, 10.7-, and 2.74-fold increases at 12, 24, and 48 h, respectively; Fig. 2, A and D). In 0.75 g/kg MEHP-treated rats, the number of CD11b+ cells increased although because of high variation this was not statistically different from any treatment at 12 h after exposure (6.0-fold increase; Fig. 2B). The number of CD11b+ was significantly increased at 24 and 48 h in the 0.75 g/kg MEHP-treated rats compared to controls (7.4- and 3.34-fold increases, respectively; Fig. 2D). In 0.5 g/kg MEHP-treated rats, the total number of CD11b+ cells were increased compared to controls at all the time points, however, not significantly (1.2-, 4.4-, and 1.9-fold increases at 12, 24, and 48 h, respectively; Fig. 2, C and D). There were no significant differences in the absolute body weight between rats within each age group (Supplemental Table S1; all the supplemental data is available online at www.biolreprod.org).

MEHP-Induced Infiltration of Newly Arrived (CD68+) Macrophages in Rats

Immunohistochemistry was utilized to further characterize the type of leukocytes infiltrating the testis (Fig. 3, A–D). Immunohistochemistry confirmed an increase in infiltrating CD68+ (newly arrived) TMs within the interstitium of the immature PND 21, 28, and 35 rat testis (Fig. 3, A–C). PND 21 control animals had more basal staining of CD68+ cells. In PND 56 rats, there were no observable differences in control and MEHP staining (Fig. 3D). At all the age groups, no leukocytes were observed within the seminiferous tubules. Negative control slides demonstrated specific binding of the primary antibody, as no background staining was observed in these controls (Supplemental Fig. S1).

Germ Cell Apoptosis Occurs Following Macrophage Infiltration in Rats

MEHP induced a significant increase in the AI in a time-dependent manner in PND 21, 28, and 35 Fischer rats (Fig. 4, A–C). These findings were consistent with previous results [25, 30]. All the peripubertal ages of rats showed a peak AI at 24 h after treatment with 1 g/kg MEHP (Fig. 4, A–C, E; PND 21, 82.45% ± 5.05%, PND 28, 98.73% ± 1.15%, vs. PND 35, 85.25% ± 7.36%). PND 21 vehicle-treated rats had higher basal AI, consistent with the timing of the first wave of spermatogenesis and normal physiological increase in germ cell apoptosis [13]. In rats (~PND 56) there was no significant change in AI at all the time points; however, at 24 h there was a trend toward significance (Fig. 4, D and E; control, 4.75% ± 1.4% vs. MEHP, 7.91% ± 0.3%). There was also a significant reduction in the relative testis weight (testis to body weight ratio) in 1 g/kg MEHP-treated peripubertal rats, while in adult rats there was no significant difference (Supplemental Table S2). There were no significant differences in the absolute body weight between rats within each age group (Supplemental Table S2).

Increased Levels of MCP-1 Occur in Testis Early after MEHP Exposure

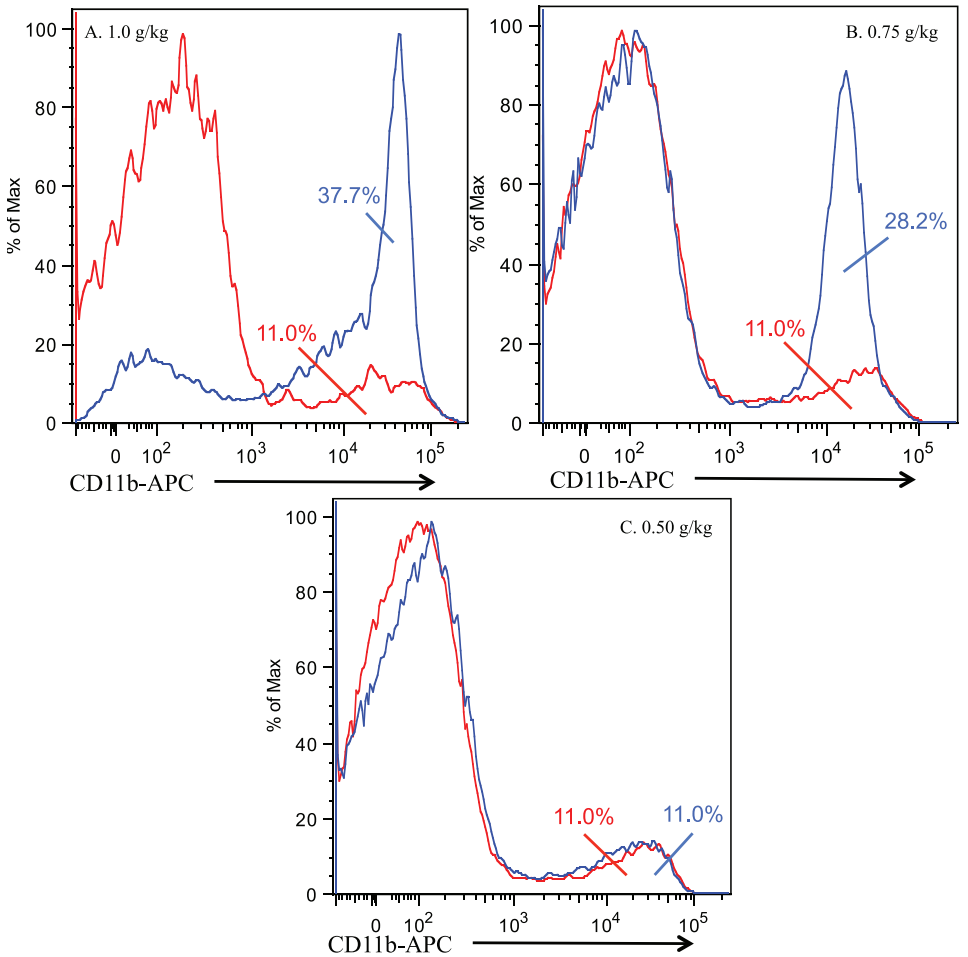
MCP-1 is the primary chemokine produced in the testis [31]. The box plot shows the median (–) and the lowest and highest data points (i.e., bottom and top of box, respectively) to convey the level, spread, and symmetry of the distributions (Fig. 5A). In all the peripubertal ages of rats, MCP-1 was significantly increased at 12 h after MEHP treatment. There were no significant differences at 24 or 48 h. MCP-1 levels decreased significantly with increasing age in all the peripubertal rats (Fig. 5). Immunofluorescence revealed that the primary source of MCP-1 in the testis is the peritubular myoid cells (PTMCs) in rats (Fig. 5, B–D). In PND 56 rats, there was no observed MCP-1 staining of the PTMCs in either treatment group (Fig. 5E). Negative control slides demonstrated specific binding of the primary antibody because no background staining was observed in these controls (Supplemental Fig. S1).

Mice Lack MEHP-Induced Infiltration of CD11b+ Cells and Robust Germ Cell Apoptosis

C57BL/6J mice have been previously established as a model of MEHP-induced Sertoli cell injury; however, it is well-known that mice are significantly less sensitive to MEHP testicular effects compared to rats [32]. Mice treated with a single 1 g/kg oral dose of MEHP showed no significant increase in interstitial CD11b+ cells as quantified by flow cytometry at all the ages (PND 21 and 28) and time points collected (12, 24, and 48 h; Fig. 6, A–C). At all the time points and ages there were no differences in T cell populations (CD4 or CD8, data not shown). The maximal AI seen in PND 28 MEHP-treated C57BL/6J mice is significantly lower than MEHP-induced germ cell apoptosis in Fischer rats (Peak AI 24% in mice [29] vs. 98% in rats; Fig. 4). No significant increase in AI was observed in PND 21 mice (Fig. 6, D and E). The level of basal germ cell apoptosis was high in control mice coinciding with the timing of the first spermatogenic wave during postnatal development. The timing of peak germ cell apoptosis in mice was not overlooked; previous reports have demonstrated that peak germ cell apoptosis is induced at 12 h or later after MEHP exposure [25, 30]. There was no significant change in the relative testis weight (testis to body weight ratio) in the immature mice (Supplemental Table S3).

DISCUSSION

An influx of circulating monocytes/macrophages into the testis has been reported in the lipopolysaccharide (LPS) and ischemia-reperfusion model of testicular inflammation [21, 33]. Inflammatory cells have been observed through histological/morphological examination of testicular cross-sections after exposure to MEHP. In the present study, we characterized the type of testicular inflammation induced by Sertoli cell injury incurred from MEHP exposure. To our knowledge, this is the first time that the type of inflammatory cells has been



D

Dose Response of CD11b+ Cells (#) After MEHP

Treatment	12h	24h	48h
CONTROL	$4.06x10^4 \pm 7.6x10^3$ a	$3.24x10^4 \pm 3.1x10^3$ a	$5.89x10^4 \pm 1.0x10^4$ a
0.5 g/kg	$4.86x10^4 \pm 9.1x10^3$ a	$1.4x10^5 \pm 7x10^4$ ab	$1.1x10^5 \pm 2.7x10^4$ ab
0.75 g/kg	$2.36x10^5 \pm 6.3x10^4$ ab	$2.4x10^5 \pm 9.6x10^4$ b	$1.97x10^5 \pm 3.2x10^4$ c
1 g/kg	$6.95x10^5 \pm 3.6x10^5$ b	$3.46x10^5 \pm 1.7x10^5$ b	$1.62x10^5 \pm 5.0x10^4$ bc

FIG. 2. Dose-dependent MEHP-induced testicular infiltration of CD11b+ cells in rats. Expression of CD11b+ cells in single cell suspension of live testicular interstitial cells after MEHP treatment at 1 g/kg (**A**), 0.75 g/kg (**B**), and 0.5 g/kg (**C**) in PND 28 rats after 12 h of exposure. Blue represent MEHP-treated rats (1 g/kg, p.o.) and red is vehicle-treated rats (corn oil, equivalent volume). The increases in the number of CD11b+ cells after MEHP treatment (1, 0.75, and 0.5 g/kg, p.o.) at PND 28 and time point are summarized in the table (**D**). Letters indicate significant differences between treatments at specified time points ($P < 0.05$, Tukey HSD; 1 g/kg: n = 6, 0.75 g/kg: n = 6, 0.5 g/kg: n = 6, control: n = 13 per time point).

characterized in the testis as a result of a specific Sertoli cell toxicant although many studies have characterized an inflammatory response in the testis due to infectious or autoimmune cause (e.g., LPS, virus, autoimmune orchitis [23, 34]).

Infectious agents cause reductions in luteinizing hormone, testosterone, and testicular interstitial fluid volume, disruption of the physical connections between the spermatogenic cells and their supporting Sertoli cells, and spermatogenic cell apoptosis [35]. Similar responses have been reported after exposure to MEHP, including breakdown of the BTB and germ cell apoptosis ([27, 36] and present study). Long-term DEHP (8 wk) exposure of male mice induces changes in the testicular immune microenvironment, including increased MHC class II-positive cells and elevated levels of IL-10 and IFN-γ testicular mRNA [37]. MEHP is reported to also have effects on Leydig cells in prepubertal rodents. However, the results are inconsistent with reports of increases, decreases, or no change in testosterone levels. These differences have been attributed to dose, route of administration, and duration of phthalate treatment [38–40]. Although, antiandrogenic mechanisms have been characterized during gestational exposure [41], a single oral dose of MEHP administered to peripubertal rats has not been convincingly associated with changes in testosterone or steroidogenic enzymes [4, 42, 43].

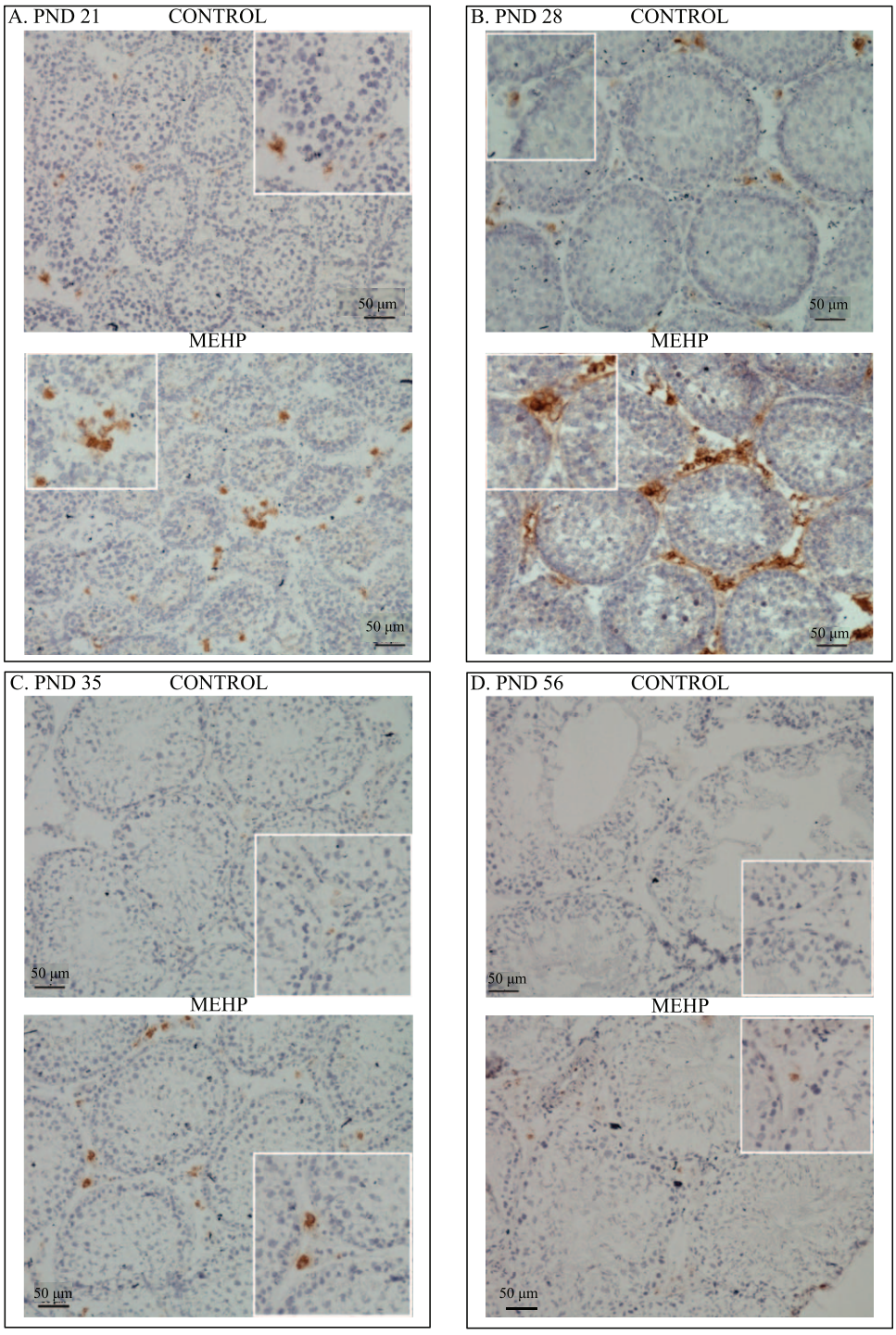
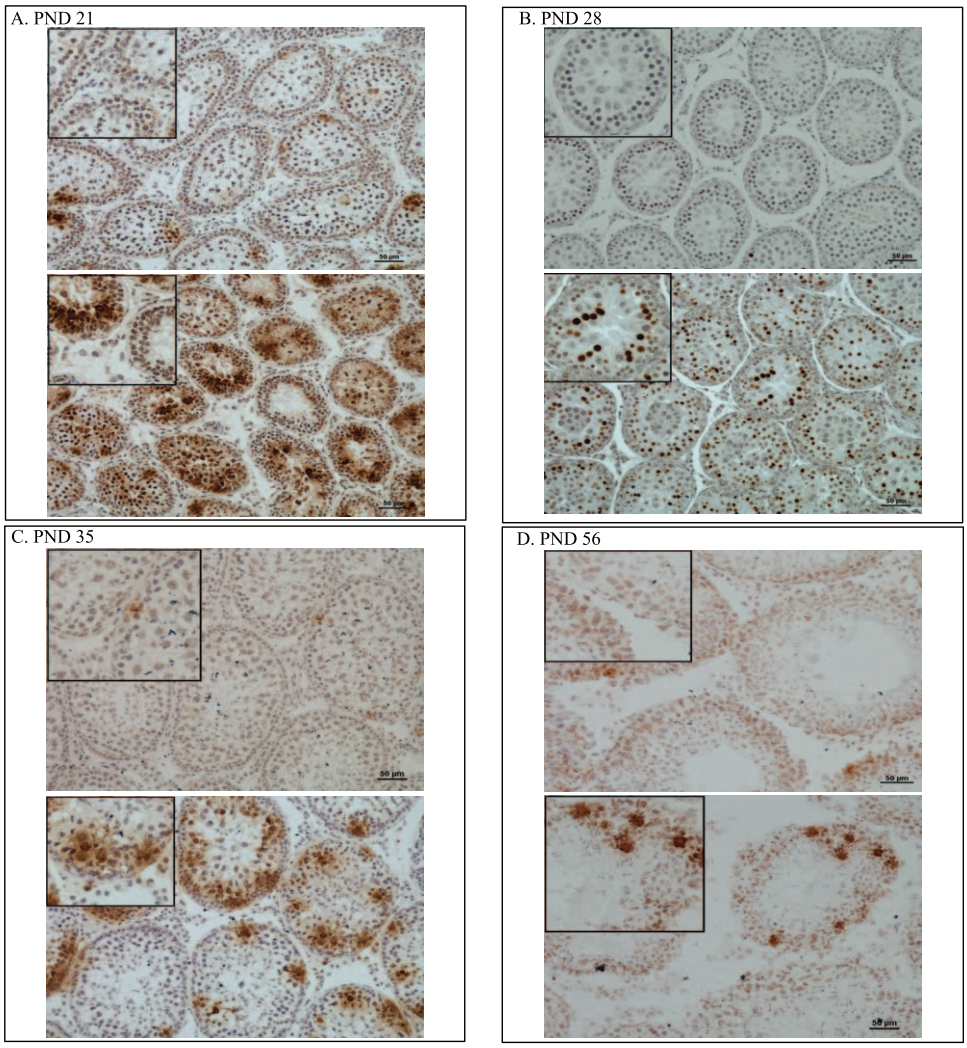


FIG. 3. MEHP-induced infiltration of newly arrived CD68+ macrophages into the testis of immature rats and PND 56 rats. Representative photomicrograph of immunostaining for CD68+ macrophages of immature rats—PND 21 (**A**), 28 (**B**), and 35 (**C**)—and mature rats—PND 56 (**D**)—treated orally for 12 h with MEHP (1 g/kg, n = 3) or vehicle (control, n = 3). Bars = 50 μm; insets magnification ×300.

In MEHP-treated peripubertal rats, the greatest infiltration of inflammatory cells occurs at 12 h posttreatment (Fig. 1, A–C) prior to the observed peak germ cell AI (Fig. 4E), thus suggesting that inflammatory cells may participate in further inducing and/or exacerbating the initiation of germ cell apoptosis. In a LPS-induced model of testicular inflammation, resident macrophages are responsible for the recruitment of newly arrived proinflammatory macrophages (CD68+) into the testis, which then induce germ cell apoptosis [35]. Similarly, MEHP induced the infiltration of CD68+ macrophages as confirmed by immunohistochemistry (Fig. 3, A–C) and extensive germ cell apoptosis in young immature rats (Fig. 4). In addition, there was no macrophage infiltration observed in adult rats (Fig. 3D), which correlates with no significant increase in germ cell apoptosis after MEHP exposure (Fig. 4). Therefore, macrophage infiltration correlates with the sensitivity to MEHP-induced germ cell apoptosis and may play a pivotal role in the mechanism.



E

APOPTOTIC INDEX
(TUNEL POSITIVE TUBULES/CROSS SECTION %)

	PND 21	PND 28	PND 35	PND 56
12 h CONTROL	15.7±8.2	0.9±0.01	3.4±0.3	3.97±2.2
12 h MEHP	61.8±3.6*	31.1±7.1*	22.7±4.4	8.09±3.6
24 h CONTROL	18.4±3.7	0.9±0.2	6.7±1.5	4.75±1.4
24 h MEHP	82.4±5.1*	98.7±1.2*	85.2±7.4*	7.91±0.3
48 h CONTROL	8.2±2.6	2.3±0.5	5.5±1.7	3.85±2.2
48 h MEHP	22.3±6.8	12.6±2.6	41.2±13.4*	5.8±0.4

FIG. 4. MEHP-induced germ cell apoptosis in rats. TUNEL staining demonstrating MEHP-induced germ cells apoptosis in immature rats. Representative photomicrograph of PND 21 (**A**), 28 (**B**), 35 (**C**), and PND 56 (**D**) rats treated orally for 24 h with MEHP (1 g/kg; lower panel) or vehicle (corn oil; top panel) demonstrate the differences in age sensitivity. Bars = 50 μm; insets magnification ×300. The AI for all the ages and time points are summarized in the table (**E**). The AI was calculated as the percentage of essentially round seminiferous tubules containing more than three TUNEL-positive germ cells in each cross-section. Asterisks designate significant differences within the age groups (mean ± SEM, $P < 0.05$, Tukey HSD; n = 3/time point/treatment).

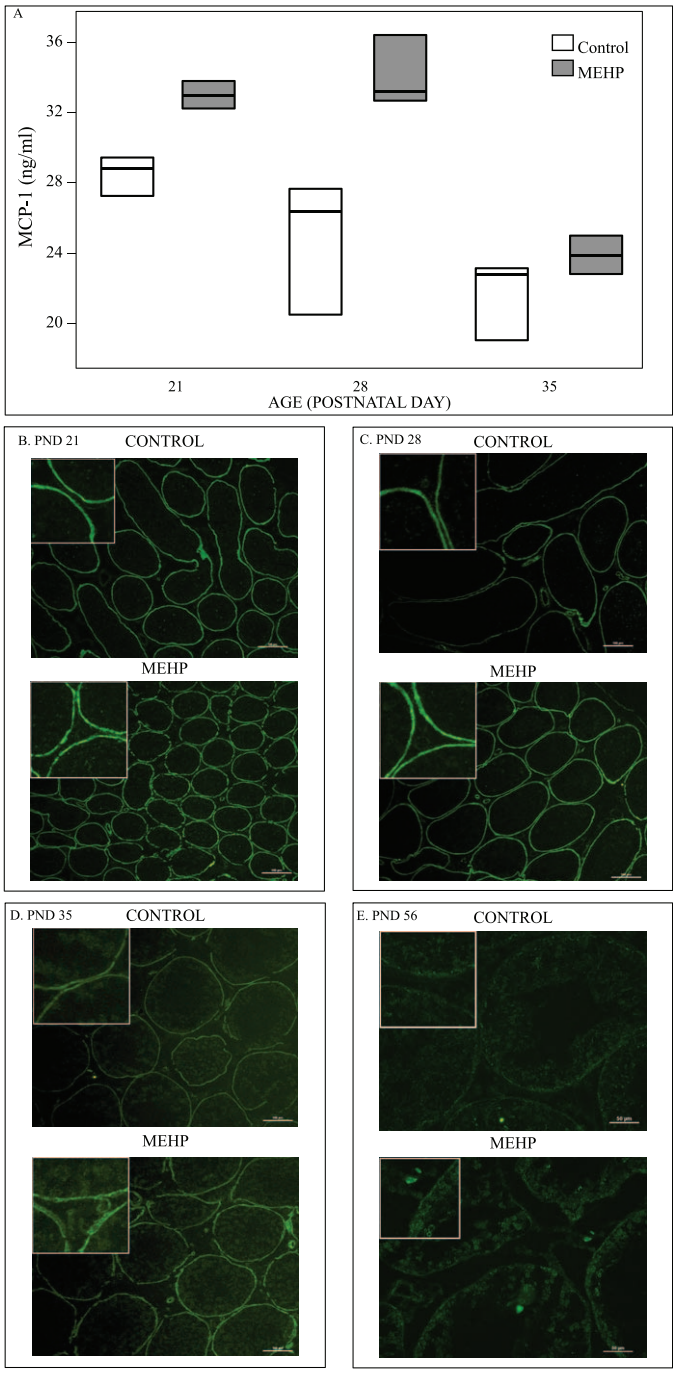
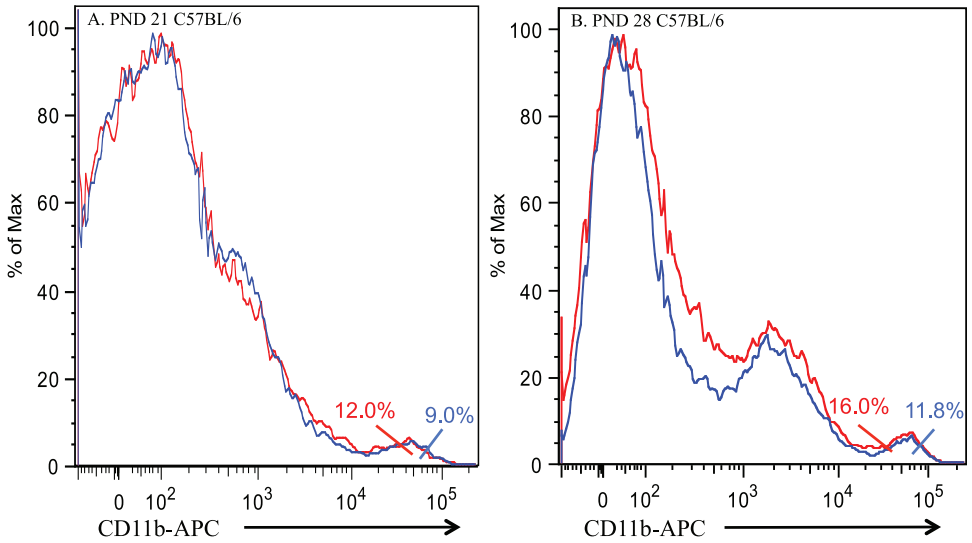


FIG. 5. Migration of macrophages into the testis after MEHP treatment is induced by MCP-1 produced by PTMCs in an age-dependent manner. The level of MCP-1 (ng/ml) within testis of PND 21, 28, and 35 rats gavaged for 12 h with MEHP (1 g/kg) or vehicle (corn oil) was quantified by ELISA. The box plot (**A**) shows the median (–) and the lowest and highest data points (i.e., the bottom and top of the box, respectively) to convey the level, spread, and symmetry of the distribution. Statistically significant differences ($P < 0.05$, ANOVA; n = 3/time point/treatment) in treatment (MEHP > control), age-dependent (PND 21 > 28 > 35) total testis concentration, and treatment by age interaction were observed. Representative photomicrograph of MCP-1 immunofluorescence staining of PND 21 (**B**), 28 (**C**), 35 (**D**), and 56 (**E**) rats treated orally for 12 h demonstrating expression specifically in PTMCs. Bars = 100 μm (**B**–**D**) and 50 μm (**E**); insets magnification ×300.

The peripubertal rat testes respond quickly to a 1.0 g/kg phthalate exposure with increased infiltration of CD68+ macrophages and germ cell apoptosis [20, 30]. Therefore, to further characterize the effects of MEHP-induced testicular inflammation, a dose response was conducted at the most sensitive age, PND 28. At this age, the lowest dose tested (0.5 g/kg) still induced an increase in CD11b+ cell infiltration; however, it did not reach statistical significance. Biphasic responses have been demonstrated in other dose-response studies that reveal changes in different genes depending on the concentration of phthalates [20]. With the currently available data, it appears that a critical level of target disruption is required for phthalates to induce testicular inflammation. Lahousse et al. [20] indicated that two chemoattractants of macrophages, Cx3cl1 and Cxcl10, and the cytokine, IL-α, gene expression are increased in prepubertal rat testes at concentrations as low as 10 mg/kg MEHP [20], a dose within reported exposure levels for sensitive populations [10]. Therefore, it is possible that a lower phthalate dose level stimulates or induces the same molecular pathways as an acute high dose. Now that this interesting phenotype has been identified, future work will need to assess if repeated lower dose exposure affects this endpoint to more accurately evaluate its relatedness to human health.

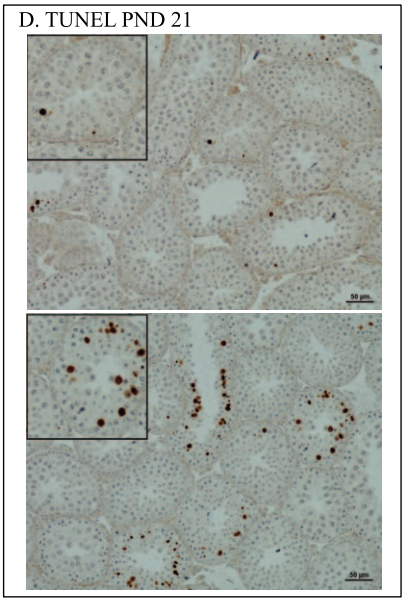
Interestingly, a single acute dose of MEHP was not observed to cause an increase in CD11b+ inflammatory cells in C57BL/6J mice, which correlates with the long-known observation that mice are significantly less sensitive to MEHP-induced germ cell apoptosis (Fig. 6). This is a well-characterized species-specific toxicity response to phthalates that has been demonstrated with di-*n*-butyl phthalate, di-(2-ethylhexyl)phthalate, and di-*n*-pentylphthalate in which rats were sensitive to the induction of germ cell apoptosis and mice were significantly more resistant [32, 44]. Although, only one strain per species was investigated in this manuscript, results of germ cell apoptosis are consistent with previously published results. Therefore, future studies will be directed to confirm that this is a species difference and not a strain difference. Previous studies have demonstrated that this is a direct effect of phthalates and not a metabolic difference between the two species [32]. Thus, infiltration of CD11b+ cells may exacerbate the toxic testicular injury due to phthalates in rats but not mice. Differences between the two rodent species in the immunoregulatory environments of the testis could explain the different sensitivity to MEHP [23].

Testicular macrophages appear in the testicular interstitium near the time of birth in rats and grow in size and number until reaching a steady-state concentration at PND 20 [45]. In the present study, we observed an age-dependent response in rats in the timing of MEHP-induced testicular inflammation. Specifically, macrophage infiltration in PND 21 rats was resolved by 24 h posttreatment, the fold increase in CD11b+ cells was much lower than in PND 28 rats, and there were significantly higher levels of MCP-1 in PND 21 rat control testis than in either the PND 28 or 35 testis (Figs. 1 and 5). In PND 56 rats, there were no changes in CD11b+ cells after MEHP exposure. In addition, control and phthalate-treated adult rats had no detectable levels of MCP-1 (Fig. 5E). Similarly, DEHP has been shown to effect immune parameters (natural killer cell activity, T-dependent antibody response, increased cytokine production) in juvenile rats (PND 10–50). This response was age-dependent, with juveniles being more sensitive to the immunotoxic effects of DEHP than adult (PND 50–90) rats [46]. Therefore, the high exposure level of children to phthalates is of great concern because this group is also more sensitive to the adverse effects of phthalates.



C C57BL/6 Fold-Change in CD11b+ Cells After MEHP

Time Point	PND 21	PND 28
12 h	0.75±0.3	1.25±0.3
24 h	0.5±0.04	0.9±0.1
48 h	0.75±0.08	0.5±0.2



E C57BL/6 Apoptotic Index PND 21

TREATMENT	TIME POINT	AI
CONTROL	12 h	6.6±1.7
MEHP	12 h	11.2±2.0
CONTROL	24 h	7.1±2.1
MEHP	24 h	11.2±4.8
CONTROL	48 h	4.0±1.8
MEHP	48 h	7.0±2.0

FIG. 6. Mice lack the MEHP-induced infiltration of CD11b+ cells and associated germ cell-induced apoptosis. PND 21 (**A**) and 28 (**B**) C57BL/6J mice were gavaged with MEHP (1 g/kg, n = 5) or vehicle (control, n = 5) for 12, 24, and 48 h. The number of testicular interstitial cells positive for CD11b+ were quantified by flow cytometry (representative histograms; **A** and **B**) and showed no differences between treatments at either age (fold-change; **C**). Representative photomicrograph of TUNEL staining of PND 21 (**D**) mice treated orally for 24 h with 1 g/kg MEHP (lower panel, n = 6) or vehicle (top panel, n = 6) demonstrate the differences in species sensitivity to severity of germ cell-induced apoptosis. Bars = 50 μm; insets magnification ×300. The AI for PND 21 mice is summarized in the table (**E**). The AI was calculated as the percentage of essentially round seminiferous tubules containing more than three TUNEL-positive germ cells in each cross-section (mean ± SEM).

MCP-1 was significantly increased in the testis of MEHP-treated juvenile rats (Fig. 5). The production of MCP-1 occurred specifically and robustly by PTMCs in immature rats (Fig. 5) [31, 47]. After MEHP treatment, MCP-1 produced by PTMCs is secreted at high levels into testicular interstitial fluid (our unpublished data), thus stimulating the infiltration of macrophages into the testis. Similarly, macrophages infiltrate the testis in response to an increase in MCP-1 after LPS treatment [47]. In adult rats, MEHP did not induce an infiltration of macrophages, which is consistent with no detectable MCP-1 expression in PTMCs. To our knowledge, this is the first time that PTMCs have been implicated in the pathogenesis of MEHP in peripubertal rats. Therefore, it is unknown if this is a direct or indirect effect of MEHP on PTMCs.

Other inflammatory cytokines released from Sertoli cells or TMs may also play an important role in the induction of macrophage infiltration as both are responsive to inflammatory stimuli [21]. Previous studies from our laboratory have demonstrated that MEHP induces a paracrine feedback loop between Sertoli cells and germ cells that initiates germ cells to undergo apoptosis via FasL-Fas signaling. Central to this pathway is the disruption of the tissue inhibitor of matrix metalloproteinase 2 (TIMP2) expression by Sertoli cells, which allows for the activation of MMP2 in the adluminal space followed by the consequent production of sTNFα. As well, MEHP- induced an increase in the production of the soluble

form of TNF-α at 1 and 3 h posttreatment in primary cocultures of rat Sertoli cells and germ cells [26]. TNF-α has been shown to increase the expression of MCP-1 from isolated PTMCs [31]. Thus, MEHP induces Sertoli and germ cells to secrete proinflammatory cytokines, which may stimulate PTMCs to secrete MCP-1 and induce macrophage infiltration. Therefore, future studies will be directed at understanding the possible contribution of other cytokines in the induction of MEHP-induced testicular inflammation and germ cell apoptosis.

Inflammation of the testis can ultimately lead to infertility. Increases in the number of CD68+ macrophages have been observed in infertile human males with germ cell arrest and Sertoli cell only syndrome [48]. These macrophages expressed genes for interleukin 1 and TNF-α [48]. The innate immune system stimulates the adaptive immune system, leading to the presentation of autoantigens of the testis and ultimately autoimmune orchitis [34, 49]. Although no changes were observed in T cell populations, an initial inflammation is followed by the activation of autoreactive lymphocytes prior to the induction of experimental autoimmune orchitis and vasectomy-induced autoimmunity [34, 49]. Investigations in our laboratory are currently underway into the effects of chronic exposure of MEHP in inducing inflammation in the testis.

In conclusion, an increased infiltration of macrophages into the juvenile rat testis was instigated after MEHP induced Sertoli cell injury. Infiltration was observed only in juvenile rat testis and occurs at time points concurrent with the robust increase in MCP-1 produced by PTMCs. Testicular macrophage infiltration corresponded with the sensitivity of young rats to MEHP-induced germ cell apoptosis and the more resistant adult rat and immature mouse. To our knowledge, this is the first study implicating a role of PTMCs and TMs in MEHP-induced germ cell apoptosis. Taken together, these results suggest that macrophage infiltration into the testis is induced prior to the peak of germ cell apoptosis, signifying a possible role of macrophages in the instigation and/or exacerbation of Sertoli cell-induced germ cell apoptosis in young rats after MEHP exposure.

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