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# Immunosuppression of T-dependent antibody responses by polycyclic aromatic hydrocarbons and arsenic in spleen cells from C57BL/6J mice

Qian Li

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**IMMUNOSUPPRESSION OF T-DEPENDENT ANTIBODY  
RESPONSES BY POLYCYCLIC AROMATIC  
HYDROCARBONS AND ARSENIC IN SPLEEN CELLS FROM  
C57BL/6J MICE**

**BY**

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**B.S. – BIOLOGICAL SCIENCES  
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**THESIS**

**Submitted in Partial Fulfillment of the  
Requirements for the Degree of**

**Master of Science  
Biomedical Sciences**

**The University of New Mexico  
Albuquerque, New Mexico**

**July, 2010**

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**B.S. — Biological Sciences, Wuhan University, China, 2006**

**M.S. — Biomedical Sciences (Toxicology), University of New Mexico, 2010**

**ABSTRACT**

Polycyclic aromatic hydrocarbons (PAHs) and arsenic are both toxic contaminants that are widely distributed in the environment. Previous studies have shown that certain levels of PAHs and arsenic exposure individually can lead to immune suppression and carcinogenesis. Co-exposures to these two classes of chemicals are commonly seen in the environment. However, no previous studies have evaluated the immunotoxicity following simultaneous exposure to both arsenic and PAHs. Therefore, the purpose of this study was to define the immunotoxicity of selected PAHs and arsenic in a murine spleen cell system, and to elucidate possible toxicological mechanisms.

Spleen cells were isolated from male C57BL/6J wild-type mice and treated with PAHs and/or arsenic. The immunotoxicological assay used in this study was the T-dependent antibody

response (TDAR) to sheep red blood cells (SRBC), measured by a direct plaque forming cell (PFC) assay. Cell viability was examined using trypan blue staining. Spleen cell viability was not altered following four days of PAHs and/or arsenic *in vitro* treatment compared to vehicle control. However, the TDAR demonstrated that the IgM antibody response was suppressed by both PAHs and arsenic in a dose-dependent manner. The PAHs and certain metabolites, including benzo[a]pyrene (BaP), BaP-7,8- diol, benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), 7,12-dimethylbenz[a]anthracene (DMBA), DMBA-3,4-diol, dibenzo[a,l]pyrene (DB[a,l]P). PAH metabolites were found to be more potent than parent compounds in producing immunosuppression. Interestingly, DB[a,l]P, a recently discovered potent carcinogenic PAH, was found to be strongly immunosuppressive following *in vitro* exposures. Sodium arsenite ( $As^{3+}$ ) was found to be more potent than sodium arsenate ( $As^{5+}$ ) in suppressing the TDAR. In addition, combination treatments of PAHs and arsenic at low doses exhibited significantly greater immunosuppressive effects than PAHs by themselves, suggesting that arsenic potentiates PAH immunosuppression. Further evidence from Western blots revealed that PAHs and  $As^{3+}$  both triggered a p53 protein up-regulation or stabilization. Combined low dose exposure of PAHs and  $As^{3+}$  demonstrated an additive p53 response that was stronger than individual treatment with PAHs or  $As^{3+}$ .

Taken together, these studies demonstrated that PAHs and arsenic suppress the TDAR in spleen cells from C57BL/6J wild-type mice, and low-dose combinations of these chemicals produced synergistic immunosuppression *in vitro*. We also demonstrated a potential role for p53 in both PAHs and arsenic induced immunotoxicity.

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## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) and inorganic arsenic are toxicologically important compounds that are widely distributed in the environment (Maier et al., 2002). PAHs are well documented immunotoxicants (Burchiel et al., 2004; Gao et al., 2008), as are certain inorganic arsenics (Burchiel et al., 2009). However, no studies have previously evaluated the effect of co-exposure to these agents on the immune system.

Environmental sources of co-exposure to these two compounds include emissions from fossil fuel combustion, cigarette smoke, and migration from hazardous waste sites. In addition, occupational exposures to arsenic in nonferrous smelters, pesticide manufacturing, or from consumption of contaminated drinking water, coupled with tobacco use, represents another important source for co-exposure to PAHs and arsenic (Maier et al., 2002; Evans et al., 2004).

### *PAHs Metabolism and Immunotoxicity*

Polycyclic aromatic hydrocarbons are a group of chemicals that are formed during the incomplete burning of coal, oil, gas, wood, garbage, or other organic substances, such as tobacco and charbroiled meat. There are more than 100 different PAHs, and they usually exist as complex mixtures in the environment (ATSDR 1996).

The major routes of human exposure to PAHs are consumption of PAHs in foods and inhalation of the compounds from tobacco smoke, wood smoke, and vehicle exhaust (Collins et al., 1998; Burchiel et al., 2004). PAHs have been found in coal tar production plants, coking plants, bitumen and asphalt production plants, coal-gasification sites, smoke houses, aluminum

production plants, coal tarring facilities, and municipal trash incinerators. For some people, the primary exposure to PAHs occurs in the workplace. Occupational workers may be exposed to PAHs by inhaling engine exhaust and by using products that contain PAHs in industries such as mining, oil refining, metal working, chemical production, transportation, and the electrical industry. PAHs have also been found in other facilities where petroleum products or coal are used or where wood, corn, or oil is burned. People living near waste sites containing PAHs may be exposed through contact with contaminated air, water, and soil.

Structures of PAHs examined in these studies are shown in Figure 1. BaP is a prototypical PAH that has been extensively studied over the past few decades. The metabolism of BaP is shown in Figure 2. BaP is metabolized by three separate enzymatic pathways (Sims et al., 1974). CYP1A1 or CYP 1B1 initiate epoxidation of BaP, followed by epoxide hydrolase hydrolyzation, forming BaP-diol, which is an unstable intermediate metabolite and substrate of CYP. The final product of BaP metabolism is BPDE, which is not a substrate of mEH because of protection by steric hindrance of nearby dihydrodiol group. Tumorigenic characteristic of BaP have been studied by a number of laboratories (Levin et al., 1982; Guengerich et al., 1991). The ultimate carcinogen of BaP is BPDE (Levin et al., 1982). The Bay region of BPDE is capable of alkylating DNA and forming bulky adducts by covalently binding to exocyclic amino groups of purines in DNA to form stable adducts (Guengerich et al., 1991; Song et al., 1998). Thus adduct formation is important in assessing and predicting carcinogenic potential. Another BaP metabolic pathway involves formation of radical cations that bind to the N7 or C8 of purines to form unstable adducts that depurinate to leave apurinic (AP) sites in DNA (Cavalieri et al., 1995). The third pathway involves the formation of BP-quinones via peroxidases (Shimada and Guengerich 2006, pathway not shown)

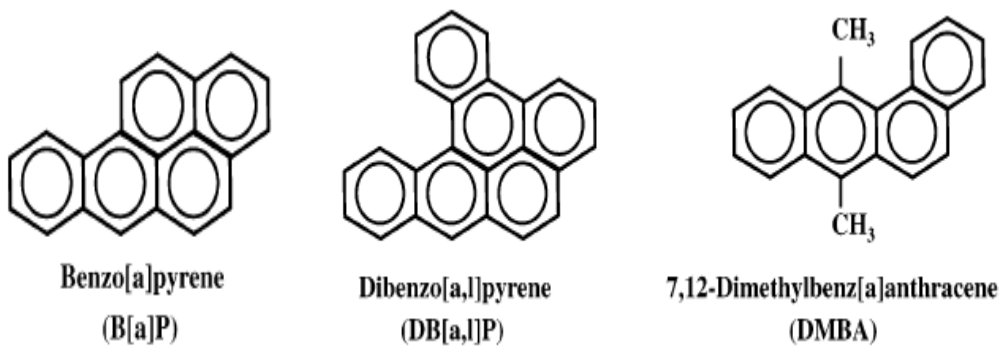
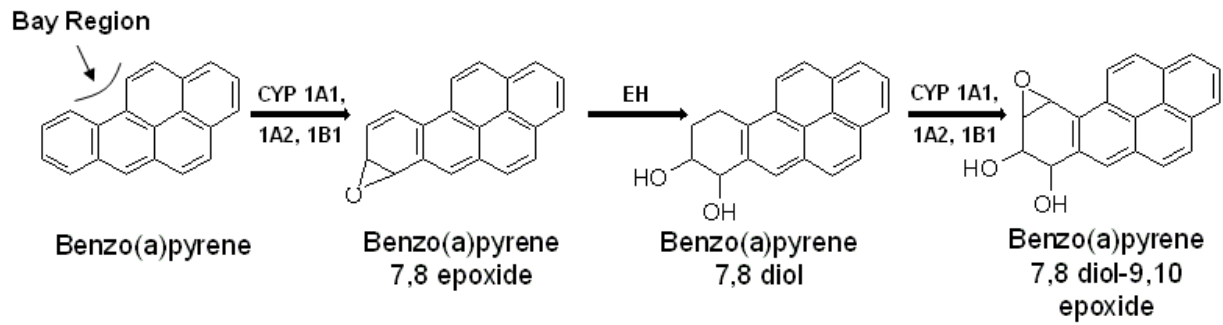


Figure 1. Chemical Structures of Selected PAHs.



**Figure 2. Schematic of Benzo[a]pyrene Metabolic Activation (Figure from “Casarett and Doull’s Toxicology” seventh edition, 2007).**

DMBA is not naturally occurring in the environment due to its photolysis and photooxidation properties (Gao et al., 2005). However, it has been studied as a model PAH extensively (Gonzalez, 2001; Gao et al., 2005). Metabolic activation of DMBA is shown in Figure 3. The two enzymes responsible for DMBA bioactivation are CYP 1B1 and microsomal epoxide hydrolase (mEH, EPHX1 gene) (Gonzalez, 2001). The final product of DMBA metabolism is DMBA-1, 2-epoxide-3, 4-dihydrodiol (DMBA-DE), which is known to be genotoxic and carcinogenic, as well as highly immunosuppressive (Cavalieri et al., 1995; Gao et al., 2005).

Dibenzo[a,l]pyrene (DB[a,l]P) is an interesting PAH because it has been discovered along with other PAHs in particulate matter (PM) air samples sources (Bergvall et al., 2007). DB[a,l]P has been determined previously as one of the most potent mouse carcinogens discovered to date (Castro et al., 2008). The metabolic pathway for DB[a,l]P bioactivation is shown in Figure 4. Previous studies on DB[a,l]P demonstrated that it shares the same CYP and EH pathways for activation with BaP and DMBA, and it shares the radical cation activation pathway with BaP and DMBA (Cavalieri et al., 1995).

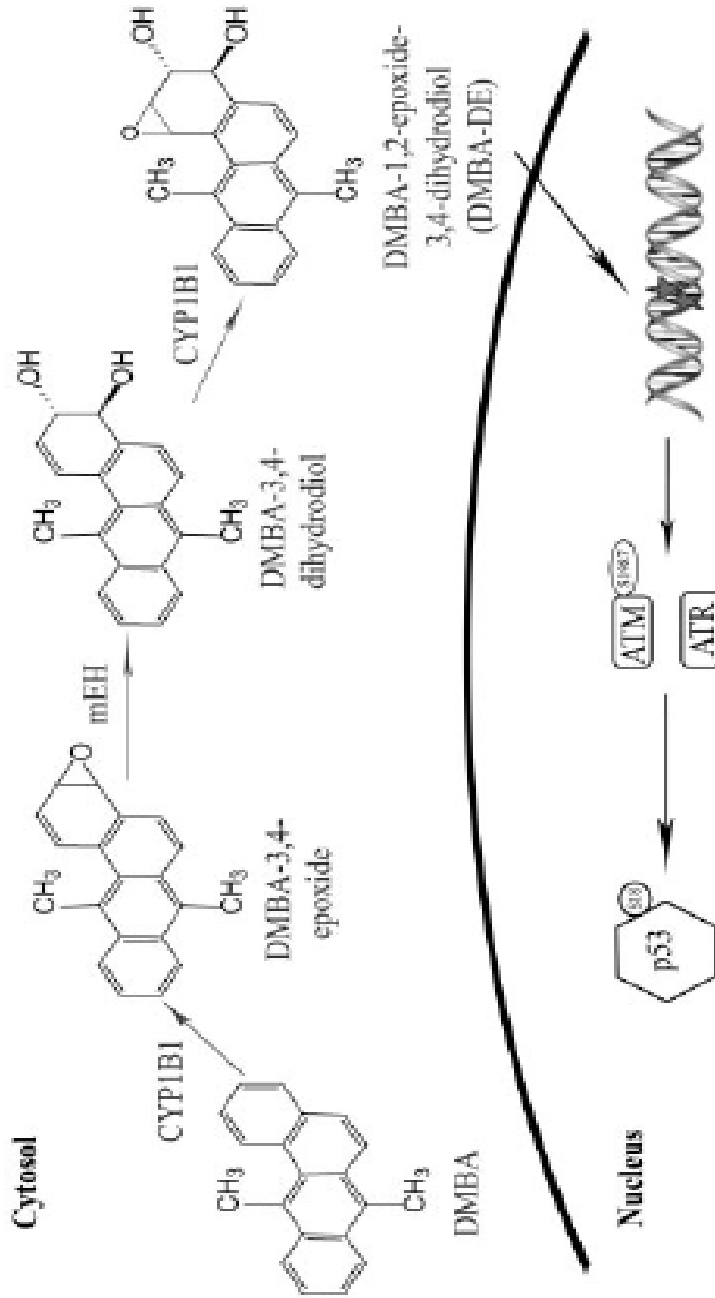
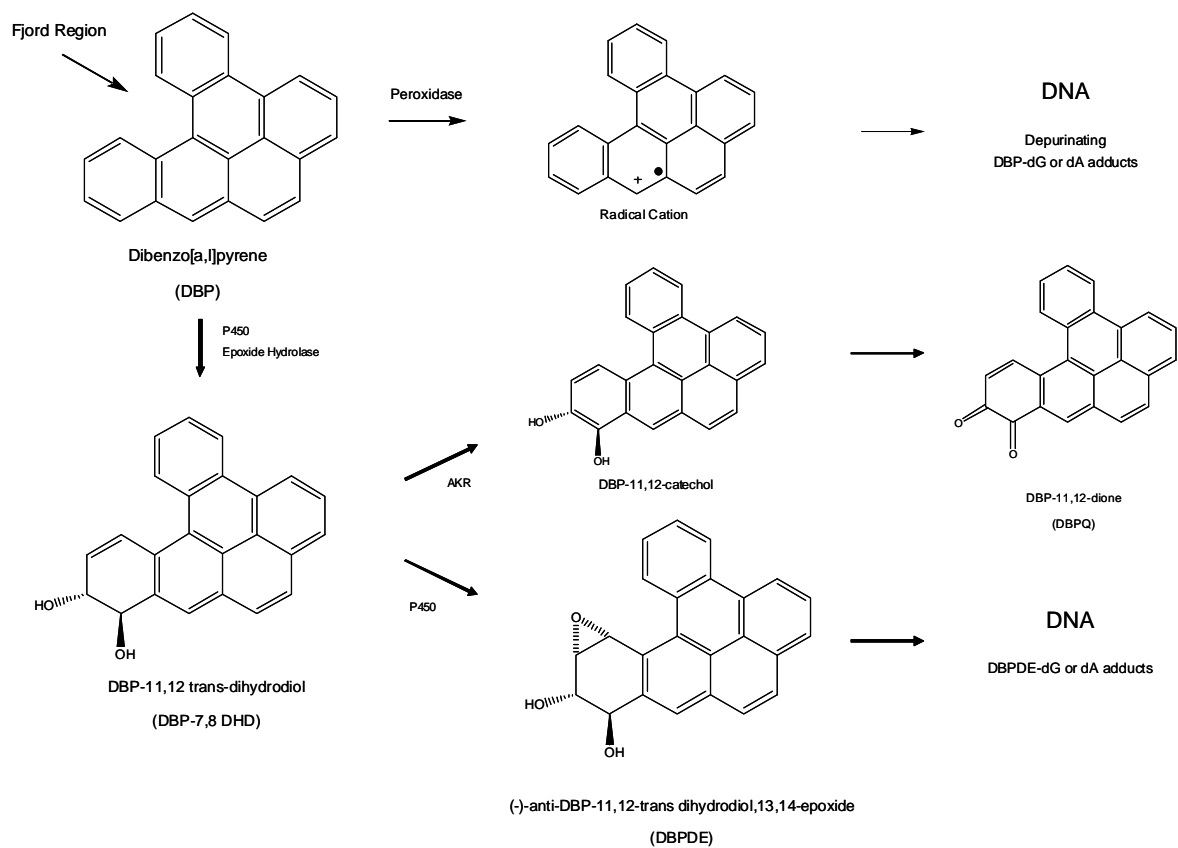


Figure 3. Schematic for DMBA metabolism and the formation of the ultimate genotoxic metabolite of DMBA, DMBA-DE (Gao et al., 2008)





**Figure 4. Schematic of Dibenzo[a,l]pyrene Metabolic Activation Pathways (Figure courtesy of Dr. David E. Williams, Oregon State University).**

### ***In Vitro and In Vivo Immunotoxicity of PAHs and their Metabolites***

The ability of PAHs to suppress immune function has been well documented in human and murine cell lines, as well as in animal models. BaP and its metabolites have been shown to induce human B cell apoptosis (Salas et al., 1998), and in human T cells, administration of BaP can cause decreased T cell proliferation to the mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) (Davila et al., 1995). Early studies showed that *in utero* BaP exposure of pregnant mice lead to severe suppression in the offspring's ability to produce antibodies (Urso et al., 1984).

DMBA is a model compound used in studying the immunosuppression of PAHs. DMBA produces extensive human T-cell suppressions (Mounho et al., 1997), as well as induces pre-B cell apoptosis and bone marrow toxicity in the mouse (Page et al., 2002). Previous studies in our lab have shown that DMBA and its metabolites persistently suppress both humoral and cell mediated immune responses in the mouse model (Burchiel et al., 1990; Burchiel et al., 1992; Gao et al., 2005). DMBA requires both CYP1B1 metabolism and the presence of p53 for the bone marrow toxicity to occur in the mouse (Heidal et al., 2002; Page et al., 2003). p53, CYP1B1 and microsomal epoxide hydrolase (mEH) are both required for spleen cell immunosuppression produced by DMBA *in vivo*. p53, CYP1B1, and mEH knockout mice are protected from DMBA induced immunosuppression (Gao et al., 2005; Gao et al., 2007; Gao et al., 2008).

BaP and DMBA have been shown to suppress ConA and PHA induced human T lymphocyte proliferation (Mounho et al., 1997). While both BaP and DMBA exposure resulted in the suppression of the TDAR to SRBC, their metabolites, BaP-7,8-diol and DMBA-3,4-diol were found to be more immunosuppressive than the parent compounds (Kawabata et al., 1987; Davila et al., 1996).

To the best of our knowledge, no research has been done to assess the immunotoxicity of DB[a,l]P. Here we predicted that it would be a potent immunosuppressant. Our studies indicated that DB[a,l]P did suppress TDAR to SRBC in murine splenocytes at very low doses, and that it is even more potent than DMBA.

### ***Arsenic Metabolism and Immunotoxicity***

Arsenic is a naturally occurring element in the earth crust. Inorganic arsenicals, arsenite and arsenate are ubiquitous in the environment. Chronic arsenic toxicity in humans has been documented in many countries worldwide, particularly in countries of Southeast Asia (Li et al., 2010). Significant arsenic exposure mostly occurs through drinking arsenic-contaminated water (ATSDR 2007).

Structures and metabolic pathways of arsenate are shown in Figure 5. Arsenate metabolism consists of reductions and methylations (Carter et al., 2003). Enzymes that catalyze the reactions are: arsenate reductase, arsenite/MMA<sup>III</sup> methyltransferase, MMA<sup>III</sup> reductase, and DMA<sup>V</sup> reductase (Aposhian et al., 2004). Previous studies considered methylation a detoxifying pathway of arsenic, but recently it has been proven that trivalent methylated arsenic metabolites MMA<sup>III</sup> and DMA<sup>III</sup> are more toxic than arsenite both *in vitro* and *in vivo* (Rossman 2003). Methylated trivalent metabolites are highly reactive and are potent inhibitors of GSH reductase and thioredoxin reductase compared with arsenite or pentavalent metabolites (Rossman 2003).

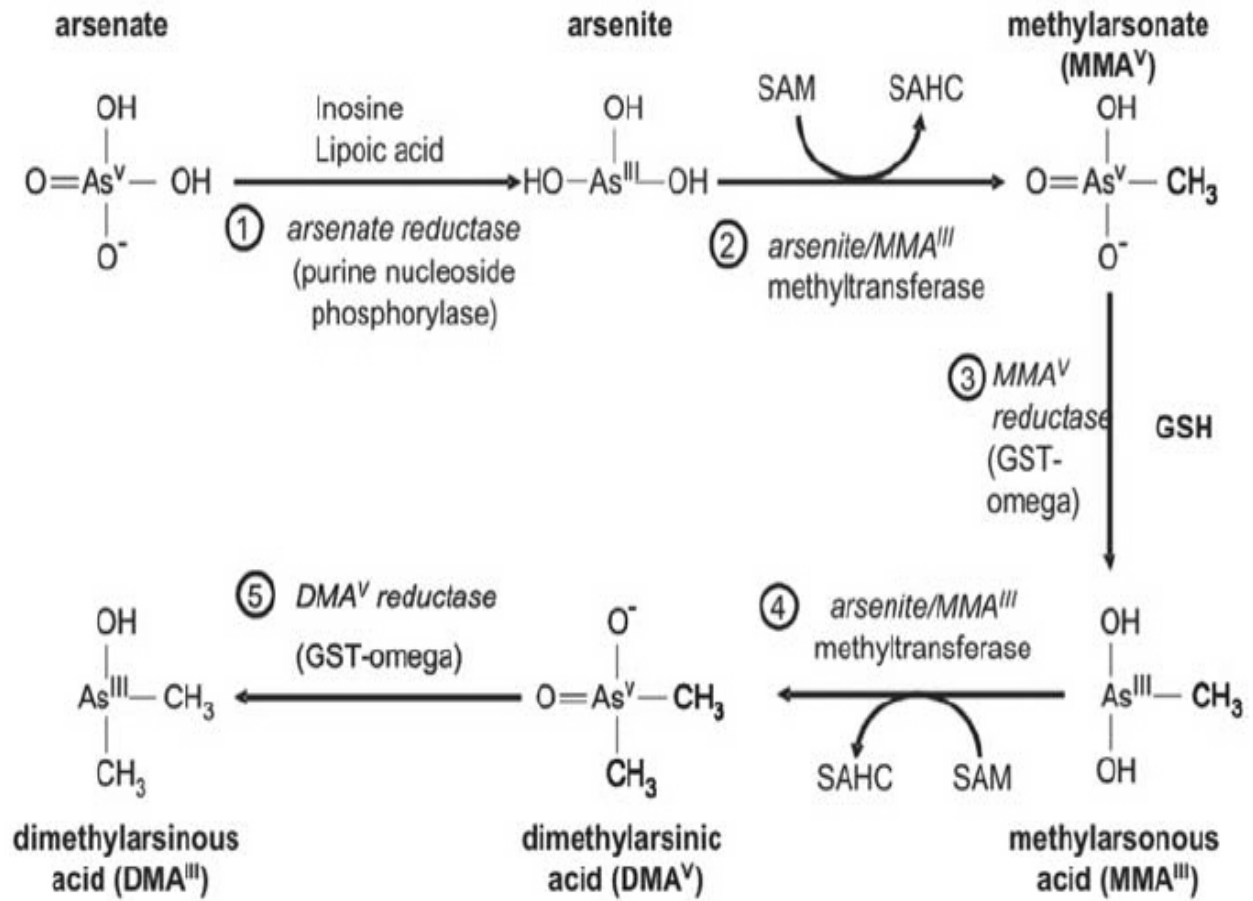


Figure 5. Known Structures and Metabolic Pathways of Arsenate in Vertebrates (Aposhian et al., 2004).

There are several studies on lung carcinogenesis using intratracheal instillation of rats and hamsters with inorganic arsenic compounds alone or with other carcinogens (Suzuki et al., 2004; Cohen et al., 2006). However, very high doses of arsenic compounds were required for very little tumor induction. The ability of various arsenicals to cause tumor promotion was also tested in rats by examining bladder tumors induced with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (Cohen et al., 2007). As a promoter for bladder (strongest response), kidney, liver and thyroid tumor, arsenite was not active. The most active promoting compound was DMA<sup>V</sup>, but MMA<sup>V</sup> and TMAO were also cancer promoters (Cohen et al., 2006).

Immunotoxic effects of arsenic have been shown in several animal models (Sikorski et al., 1989; Burns et al., 1993; Burchiel et al., 2009) as well as in humans (Soto-Peña et al., 2006). B6C3F1 female mice exposed to a single dose of gallium arsenide (GaAs) exhibited an inhibition of T cell proliferation and macrophage activity, along with a reduction in IgM and IgG production (Burns et al., 1993). Also, GaAs-treated mice demonstrated a 50% decrease in CD4<sup>+</sup> splenic cells after 24 h (Sikorski et al., 1989). A two-week inhalation exposure to arsenic trioxide in C57Bl/6J mice significantly suppressed the TDAR response by 70% (Burchiel et al., 2009). Exposure to sodium arsenite in drinking water has been reported to result in a down-regulation of several cytokines in children living in arsenic contaminated areas in Mexico (Soto-Peña et al., 2006).

Previous *in vitro* studies on the genetic toxicology of arsenite have shown that this agent is not a potent mutagen and does not react directly with DNA (Snow et al., 2005; Klein et al., 2007). However, at very early times after exposure, As<sup>3+</sup> was found to activate NADPH oxidase activity through a Ras-GTPase mechanism, which creates oxidative damage to cells. Reactive oxygen species (ROS) and nitric oxide synthase (NOS) were found to be increased in human

keratinocytes treated with arsenite (Shi et al., 2004; Qin et al., 2008). A recent study in human keratinocytes demonstrated that low concentration of sodium arsenite inhibited Poly (ADP-ribose) Polymerase-1 and interfered with the DNA repair machinery (Ding et al., 2009).

### ***Potential Arsenic-PAH Interactions***

While arsenic alone is not a potent carcinogen, recent studies show that it plays a role in PAH and UVA induced carcinogenesis (Maier et al., 2002; Evans et al., 2004). Recent studies showed that arsenite co-treatment enhanced the formation of stable BaP–DNA adducts and this increase in genotoxicity was found to be cytochrome p450 1A1 (CYP1A1)-dependent. Evidence also showed that glutathione depletion enhanced the potentiating effect of arsenite on formation of adducts.

Based on the fact that immunosuppression has been demonstrated by PAHs and arsenic separately, we were interested in exploring the immunotoxic effects by combination of these two agents. Both arsenic and PAHs exposure have been shown to produce genotoxicity and oxidative stress. We believe that genotoxicity may be the major pathway for both arsenic and PAH induced immunosuppression. Therefore, the present studies were designed to characterize the immunosuppressive properties of selected PAHs and arsenic and to determine whether these two classes of chemicals produce synergistic or additive immunosuppression. The second goal of this study was to determine whether the immunosuppression by arsenic and PAHs is p53-dependent. Finding that PAH and arsenic alter p53 alone or in combination would provide initial evidence for a genotoxic mechanism of action for these agents.

## HYPOTHESIS AND SPECIFIC AIMS

### *Hypothesis*

The hypothesis tested in this study is that arsenic and PAHs co-exposures potentiate immunosuppression in C57BL/6J mouse splenocytes. The current study also hypothesized that p53 is associated with the immunosuppression produced by arsenic and PAHs.

### *Specific Aim 1*

**Develop an *in vitro* model to examine PAHs and arsenic induced immunosuppression given alone or in combination.**

### *Specific Aim 1a*

Determine and establish dose responses for TDAR immunosuppression produced by sodium arsenite, sodium arsenate, BaP, DMBA, DB[a,l]P, and selective PAH metabolites. This was achieved by *in vitro* treatment of wild type mouse spleen cells using various doses of PAHs (listed above), arsenite and arsenate, followed by examination of the TDAR assay to test immunosuppression.

### *Specific Aim 1b*

Determine if co-exposure to arsenic and PAHs potentiates the immunosuppression compared to PAH alone. This was done by combining the treatments of arsenic and PAHs in wild type mice spleen cells and testing the immunosuppression using TDAR.

### ***Specific Aim 2***

**Study the mechanism of immunosuppression produced by Arsenic and PAHs alone or in combination in mice splenocytes.**

#### ***Specific Aim 2a***

Determine if the arsenic and PAH immunosuppression is p53 dependent. This was done by combining the *in vitro* treatments of arsenic and PAHs in p53 knockout mice spleen cells and testing the immunosuppression by TDAR to see if p53 null mice are protected from arsenic-PAHs induced immunosuppression.

#### ***Specific Aim 2b***

Study p53 protein regulation in response to arsenic and PAH treatments. This was done by examining p53 protein level in mouse spleen cells treated with arsenic and/or PAHs using Western blot analysis as described in the Material and Methods.



## MATERIALS AND METHODS

### *Chemicals and reagents.*

Sodium arsenite, sodium arsenate, benzo[a]pyrene, 7,12-dimethylbenz[a]thracene (DMBA) and dibenzo[a,l]pyrene (DB [a,l]P) were purchased from Sigma-Aldrich (St. Louis, MO). Benzo[a]pyrene-*trans*-7,8dihydrodiol, Benzo[a]pyrene-*trans*-7,8dihydrodiol-9,10-epoxide and 7,12-Dimethylbenz[a]-anthracene-*trans*-3,4-dihydrodiol were obtained from National Cancer Institute Chemical Repository (Kansas City, MO). Sodium arsenite was dissolved in culture grade water, PAHs were dissolved in culture grade anhydrous dimethylsulfoxide (DMSO; Sigma, St. Louis, MO). Both water and DMSO served as the solvent control. The final concentrations of water and DMSO in all cell cultures were 0.1%. This concentration was determined to be without measurable effect on cellular response. Cell culture materials were from Sigma-Aldrich and Invitrogen (Grand Island, NY).

### *Animals.*

Male wild-type C57BL/6J mice (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). p53-null mice breeders were purchased from The Jackson Laboratory. These knockout mice were bred in our Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility under an Institutional Animal Care and Use Committee-approved protocol. All knockout mice were genotyped using a polymerase chain reaction genotyping method with DNA isolated from tail snips (Jacks et al., 1994; Miyata et al., 1999). No animals bearing tumors were used in these

studies. In all of the experiments, mice were euthanized by CO<sub>2</sub>, followed by spleen cell isolation, described as below. All mice were used at the age of 10-14 weeks old. Mice spleen weights were recorded at the time of euthanasia.

### ***Spleen cell preparation.***

Single cell suspensions were prepared and combined from three individual mice per treatment group. Spleen cells were harvested as described previously (Gao et al., 2005). In brief, spleen cells were isolated in RPMI 1640 complete medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 Units/ml penicillin, and centrifuged at 280x g for 10 min. Cell pellets were resuspended and maintained in RPMI 1640 complete medium on ice. Viable spleen cell counts were obtained by the trypan blue (Sigma Chemical Co., St. Louis, MO) exclusion method using a hemacytometer.

### ***In vitro treatment and plaque-forming cell assay.***

Mouse spleen cells collected sterilely (4 x 10<sup>6</sup> cells/ml, 0.5 ml) were treated with arsenic and/or PAHs and cultured for four days with 0.5 ml of twice washed 1% sheep red blood cells (SRBC) (Colorado Serum, Denver, CO) in 48-well, flat-bottomed plates (Corning Glass, Corning, NY) with PFC media [RPMI 1640 medium with 10% heat inactive fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 50 µM 2-mercaptoethanol (GIBCO, Grand Island, NY), 1 mM sodium pyruvate (GIBCO, Grand Island, NY) and 50 µg/ml gentamycin (GIBCO, Grand Island, NY)]. The plates were placed in a humidified, 37°C, 5% CO<sub>2</sub> incubator. PFC media without SRBC were added into the spleen cells as control. Quadruplicate cultures were run for each treatment with SRBC. Meanwhile, a control plate for checking cell viability is also

set up. Four days later, a glass slide modification of Jerne and Nordin (1963) PFC assay was performed. Briefly, the immunized spleen cells were collected from individual cultures into glass tubes, and washed twice with PFC media. Then 50  $\mu$ l 50% SRBC were then added to each glass tube. These tubes were placed in a 43°C constant temperature water bath with 400  $\mu$ l 0.8% Seaplaque agarose (Intermountain Scientific, Kaysville, UT). SRBC were added to the tubes and one slide was used for each culture (quadruplicate) to determine the PFC response. The mixture of spleen cells and SRBC was poured onto 3x1x1 mm, 0.15% Seaplaque agarose precoated microscope slide and allowed to cool. The slides were inverted on custom-made slide trays. The slides were incubated for 1.5 h at 37°C in a humidified without CO<sub>2</sub> incubator. Guinea pig complement (Colorado Serum, Denver, CO) in Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium (1:20) was used to flood the slides on each tray. Following additional 2 h incubation at 37°C, the numbers of anti-SRBC plaque-forming cells (PFC) per culture were identified by dissecting microscope. The data are presented as the number of PFC/culture ( $2 \times 10^6$  cells per culture on day 0).

***In vitro treatment and whole cell lysate preparation.***

Mouse spleen cells were collected sterilely as described above ( $6 \times 10^6$  cells/ml, 1ml) in PFC media [PMI 1640 medium with 10% heat inactive fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml penicillin with 100  $\mu$ g streptomycin sulfate (Lonza, Walkersville, MD) and 50  $\mu$ g/ml gentamycin (GIBCO, Grand Island, NY)] in 48-well, flat-bottomed plates. Cells were treated with arsenic and/or PAHs and cultured for 4 h, 8h and 18h in a humidified, 37°C, 5% CO<sub>2</sub> incubator. To prepare the whole lysate, spleen cells from each treatment were collected in a 15 ml centrifuge tube, centrifuged at

280 x g for 10 min at 4°C. The supernatants were discarded, and the pellets were resuspended in 2 ml ammonium chloride lysine solution (10 X; 1.5 M ammonium chloride, 100 mM sodium bicarbonate, 10mM disodium EDTA, pH 7.4). Samples were held at room temperature for 10 min, followed by washing twice with DPBS. The pellets were resuspended in 200 µl RIPA buffer (50mM Tris, 150mM sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton X100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and Roche Complete Protease Inhibitor Tablet, pH 7.4) and transferred to 1.5 ml microfocentrifuge tubes. Suspensions were held on ice for 10 min, sonicated on ice for 10 sec, centrifuged at 18,000 x g for 10 min at 4°C. The supernatant were collected in a 0.65 ml microcentrifuge tube. Total protein concentrations were determined using Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA)

#### ***Western blot analysis.***

Total p53 and β-actin expression were analyzed as previously described (Gao et al., 2005). Briefly, 100 µg of whole cell lysate was heated at 95°C for 5 min with 6X sample buffer (0.35M Tris, pH6.8, 30% Glycerol, 10% SDS, 0.6M dithiothreitol, 0.012% bromphenol blue). Samples were separated by SDS polyacrylamide gel electrophoresis using a 10% resolving gel with a 5% stacking gel and a mini-PROTEIN 3 cell system (Bio-Rad Laboratories). After 1 h electrophoresis at 180 Volts, protein was transferred to nitrocellulose membranes (0.45 µm; Bio-Rad Laboratories) for 1 h using a constant 300 milliamps current. Nonspecific binding was blocked by incubating membranes in 5% (w/v) Nonfat Dry Milk (Bio-Rad Laboratories) in Tris-buffered saline containing Tween 20 [BS/T; 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween 20] at room temperature for 1 h. Incubation was followed by three 5 min TBS/T washes;

membranes were then incubated with a p53 1C12 antibody (1:1000; Cell Signaling Technology Inc., Danvers, MA) in 5% bovine albumin BSA (Sigma-Aldrich) in TBS/T at 4°C overnight. After washing with TBS/T, membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (1:2000; Cell Signaling Technology Inc.) for 1 h at room temperature in 5% BSA in TBS/T. The protein bands were detected using a SuperSignal<sup>®</sup> West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) and visualized on a Kodak Image Station 4000 mm (Eastman Kodak, Rochester, NY). The protein molecular weight was determined by comparison with Precision Plus Protein Western C standards (Bio-Rad Laboratories). After detection of p53 protein, the membranes were stripped using Gentle ReView Buffer (Amresco Inc., Solon, Ohio) in a 37°C water bath for 30 min and were then probed with  $\beta$ -actin antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA).

### ***Statistical analysis.***

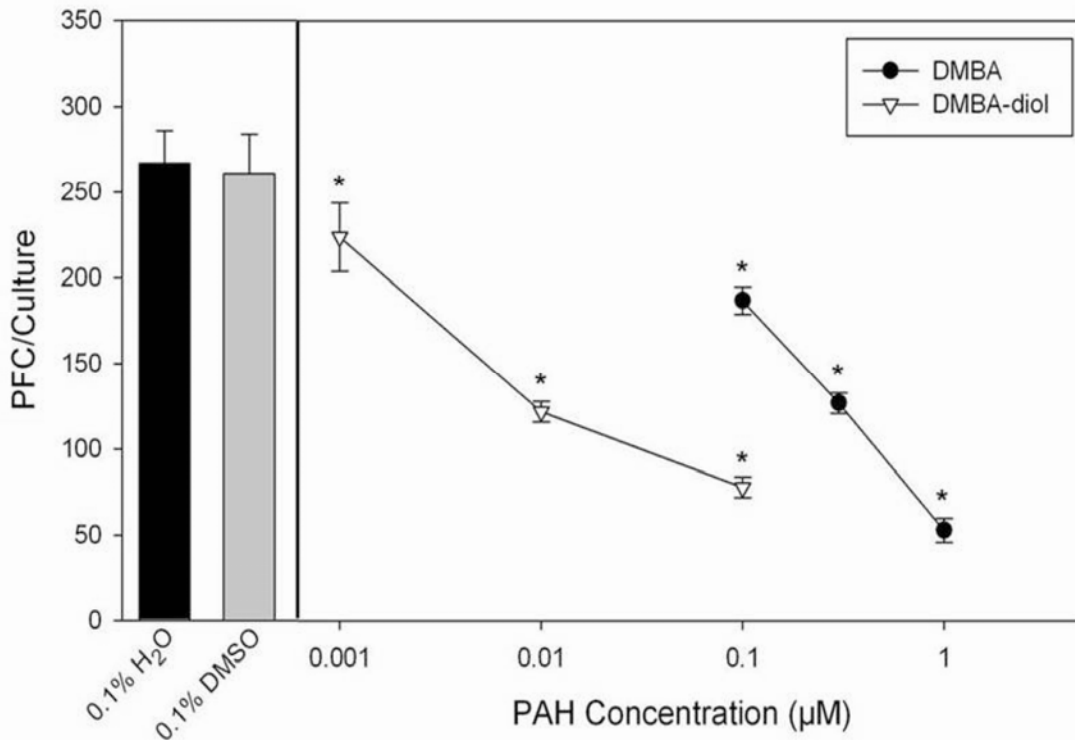
All of the data reported in this paper were analyzed by SigmaStat software (Jandel Scientific, San Rafael, CA). The statistical differences were determined by one-way analysis of variance (ANOVA). A p-value of <0.05 will be considered significant. Data is reported as the average  $\pm$  SEM for replicate cultures (as indicated) which measures the inter-culture variability of treatments rather than the inter-animal variation in response.

## RESULTS

### *SPECIFIC AIM 1*

#### *DMBA and DMBA-diol Suppress the Spleen Cell TDAR in C57BL/6J mice.*

Our laboratory has previously shown that TDAR is a very sensitive method to examine immunosuppression that xenobiotics may produce *in vitro* and *in vivo* (Burchiel et al., 1990; Burchiel et al., 2009). To characterize the spleen cell humoral immune response following DMBA or DMBA-diol treatments, the TDAR response to SRBCs was examined *in vitro* using PFC assay (Figure 6). Both DMBA and DMBA-diol produced concentration-dependent suppression in the TDAR response in C57Bl/6J mice spleen cells. At the lowest concentration of DMBA tested (0.1  $\mu$ M) a 30% suppression in TDAR compared to DMSO vehicle control was detected, whereas DMBA-diol produced nearly the same level of suppression at a concentration of 0.001  $\mu$ M. These results indicate that DMBA-diol is 30-100 times more potent than the parent compound in producing immunosuppression in our system.



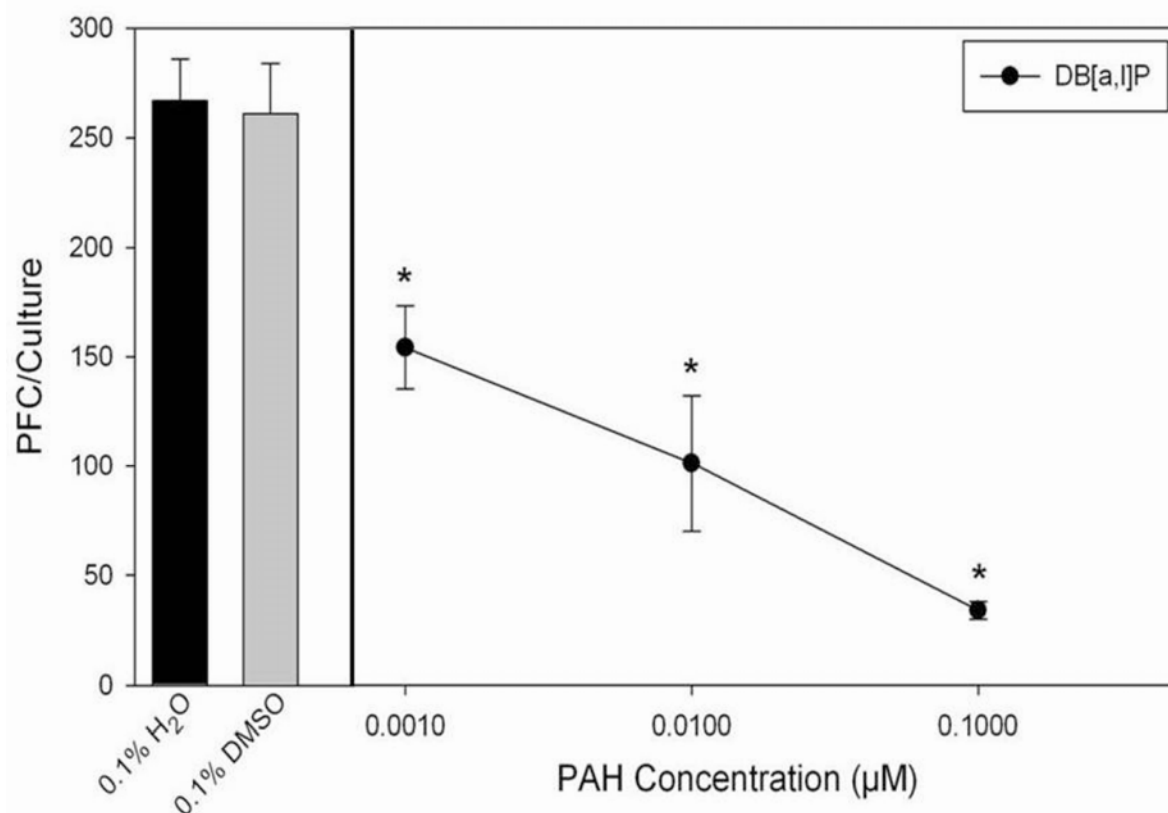
**Figure 6. DMBA and DMBA-diol Suppress the Spleen Cell TDAR in C57BL/6J mice.**

Spleen cells were immunized *in vitro* at the same time of treatment with DMSO, DMBA, or DMBA-diol. The number of antibody producing spleen cells was determined by TDAR PFC assay as described in the Materials and Methods. No cytotoxicity compared to control was observed in these cultures. Data are for a pool of three mice spleens that were assayed in quadruplicate culture. Data are shown as mean  $\pm$  S.E.M. \* indicates statistically significant differences from 0.1% DMSO control ( $p < 0.05$ ).

***Immunosuppressive Effect of DB[a,l]P on the Spleen Cell TDAR in C57Bl/6J mice.***

DB[a,l]P has been shown to share several chemical properties and has a reactive biological structure similar to DMBA (Melendez-Colon et al., 1999). The immunotoxicity of DB[a,l]P has not been previously reported. We found that DB[a,l]P produced a concentration-dependent suppression of the TDAR response. In fact, DB[a,l]P was even more potent than DMBA (Figure 7). Following DB[a,l]P exposure of murine spleen cells in culture for four days, 0.001  $\mu$ M DB[a,l]P produced 40% suppression of the TDAR compared to the 0.1% DMSO control cultures. These results suggest that DB[a,l]P may be up to 100 times more potent than DMBA in producing immunosuppression.



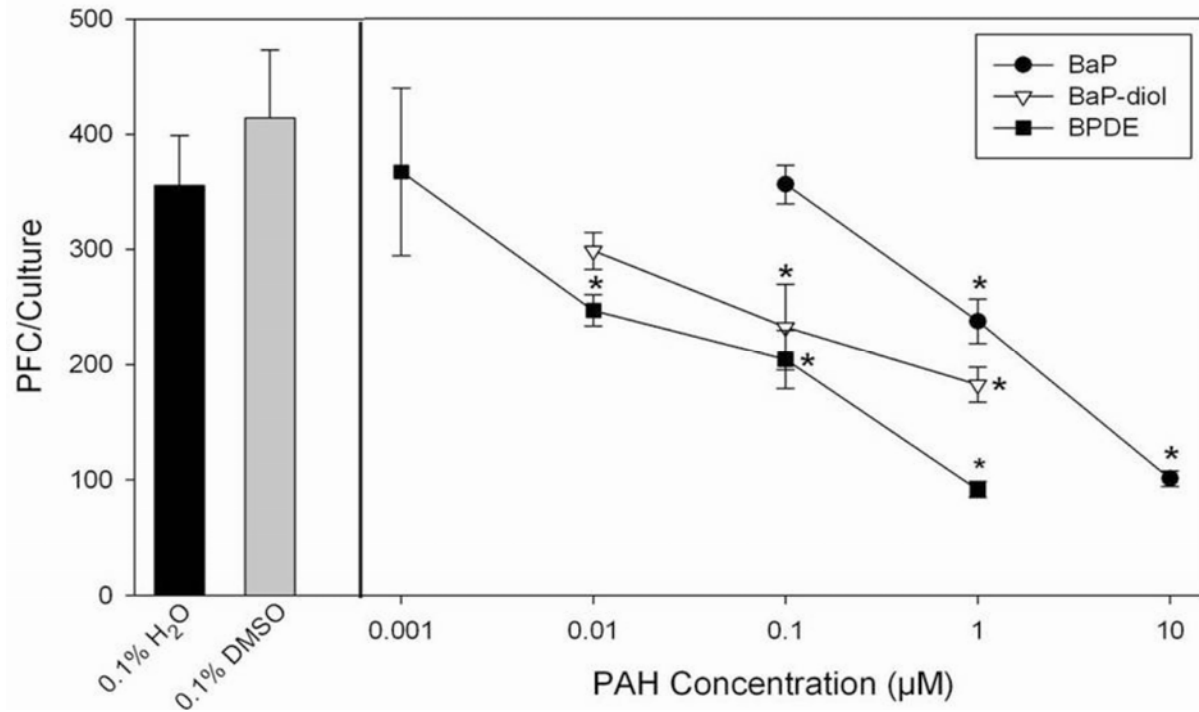


**Figure 7. Immunosuppressive Effect of DB[a,l]P on the Spleen Cell TDAR in C57Bl/6J mice.**

Spleen cells were immunized *in vitro* with SRBC, and treated with DMSO or DB[a,l]P. The number of antibody producing spleen cells was determined by a TDAR PFC assay as described in the Materials and Methods. No cytotoxicity compared to control was observed in these cultures. Data for a pool of spleen cells from three mice cultured in quadruplicate are shown as mean  $\pm$  S.E.M. Statistically significant differences compared with 0.1% DMSO are indicated (\*,  $p < 0.05$ ).

***Immunosuppressive Effect of BaP, BaP-diol and BPDE on the Spleen Cell TDAR in C57BL/6J mice.***

Because BaP is an important environmental PAH that is bioactivated by cells to form BaP-diol and BPDE, we compared the concentration-dependent effects of these three agents (Figure 8). These PAHs exhibited different potencies for suppression of the TDAR. At a concentration of 1  $\mu$ M, BaP produced about 50% suppression of the TDAR, whereas BaP-diol was 10-fold more immunosuppressive than BaP, and BPDE was 100 times more suppressive than BaP. These observations agree with our current understanding that BaP needs to be metabolized by CYPs (1A1 and 1B1) and microsomal epoxide hydrolase (EPHX1) to exert its immunotoxicity.

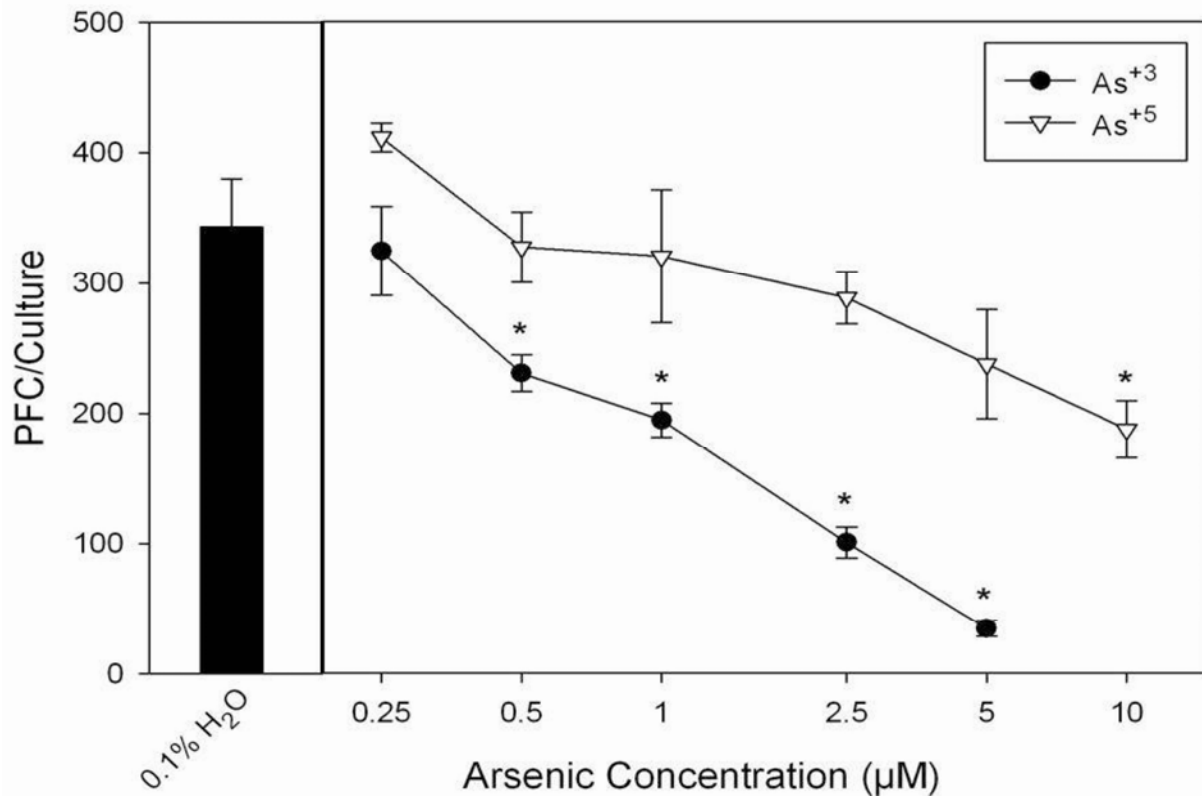


**Figure 8. Immunosuppressive Effect of BaP, BaP-diol and BPDE on the Spleen Cell TDAR in C57BL/6J mice.**

Spleen cells were immunized *in vitro* with SRBC, and treated with DMSO, BaP, BaP-diol, or BPDE. The number of antibody producing spleen cells was determined by a modified Jerne and Nordin PFC assay as described in the Materials and Methods. No cytotoxicity was observed in these cultures compared to control culture. Data for a pool of three mouse spleens, assayed in quadruplicate, are shown as mean  $\pm$  S.E.M. Statistically significant differences compared with 0.1% DMSO are indicated (\*,  $p < 0.05$ ).

***Immunosuppressive Effects of NaAsO<sub>2</sub> and Na<sub>2</sub>HAsO<sub>4</sub> on the Spleen Cell TDAR in C57Bl/6J mice.***

Sodium arsenite and sodium arsenate immunotoxicity in C57Bl/6J mice spleen cells were measured using the TDAR and PFC assay (Figure 9). They both had immunosuppressive effects in a dose-dependent manner. Sodium arsenite (As<sup>3+</sup>) was found to suppress the TDAR at concentrations as low as 0.5 µM. Sodium arsenite was found to be at least 10-fold more suppressive to the TDAR than sodium arsenate (As<sup>5+</sup>).

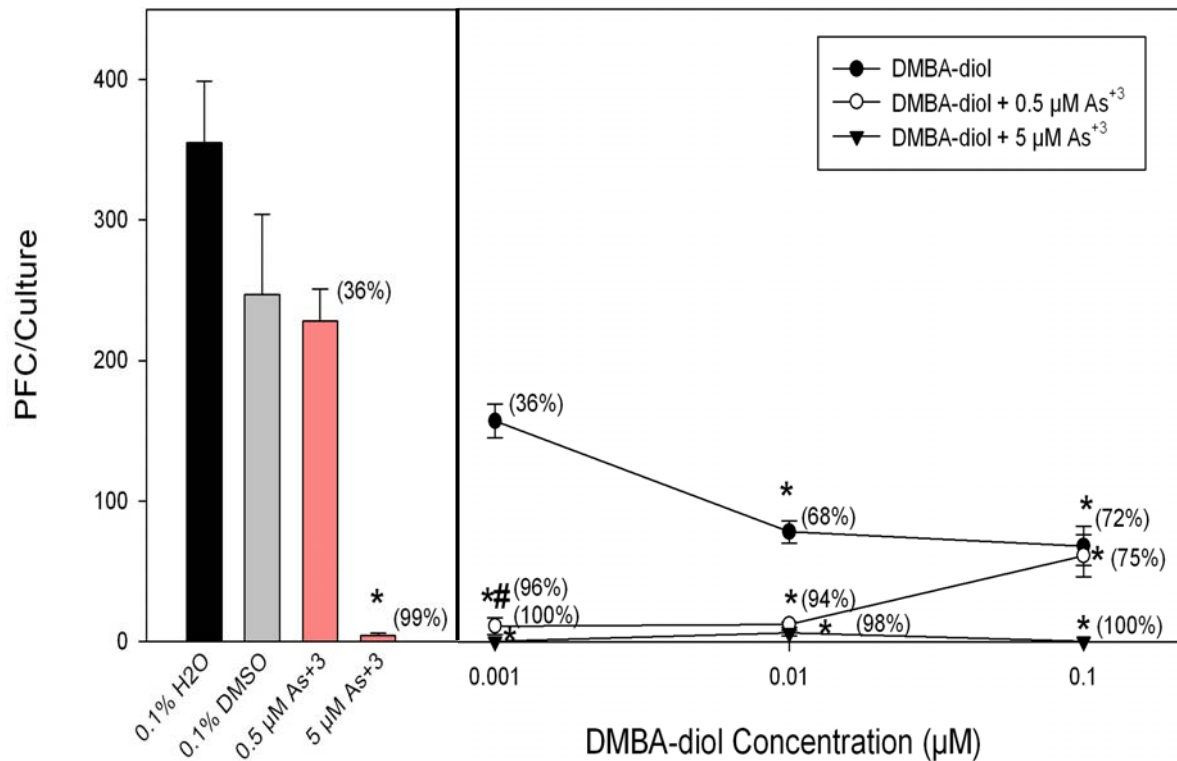


**Figure 9. Immunosuppressive Effects of As<sup>3+</sup> and As<sup>5+</sup> on the Spleen Cell TDAR in C57Bl/6J mice.**

Spleen cells were immunized in vitro with SRBC and treated with H<sub>2</sub>O (arsenic vehicle) or As<sup>3+</sup>, As<sup>5+</sup>. The number of antibody producing spleen cells was determined using a modified Jerne and Nordin PFC assay as described in the Materials and Methods. No cytotoxicity was observed in these cultures compared to control. Data for a pool of three mouse spleens those were assayed in quadruplicate, shown as mean  $\pm$  S.E.M. Statistically significant differences compared with 0.1% H<sub>2</sub>O are indicated (\*,  $p < 0.05$ ).

***Immunosuppressive Effect of Combining As<sup>3+</sup> and DMBA-diol on Splenocytes TDAR from C57Bl/6J mice.***

In order to examine the effect of co-exposure to arsenite and DMBA-diol, 0.5  $\mu\text{M}$  and 5  $\mu\text{M}$  doses of sodium arsenite were chosen to combine with DMBA-diol at 0.001  $\mu\text{M}$ , 0.01  $\mu\text{M}$  and 0.1  $\mu\text{M}$  respectively. Spleen cells were treated and immunized with SRBC at the same time. The TADR PFC assay was used to measure immunosuppression. As shown in Figure 10, DMBA-diol produced a dose-dependent immunosuppression of the TDAR PFC response. The 0.5  $\mu\text{M}$  dose of sodium arsenite in the cell cultures greatly potentiated immunosuppression produced by DMBA-diol by itself at 0.001  $\mu\text{M}$ .



**Figure 10. Immunosuppressive Effect of Co-exposure to As<sup>3+</sup> and DMBA-diol on C57Bl/6J mice Splenocytes TDAR.**

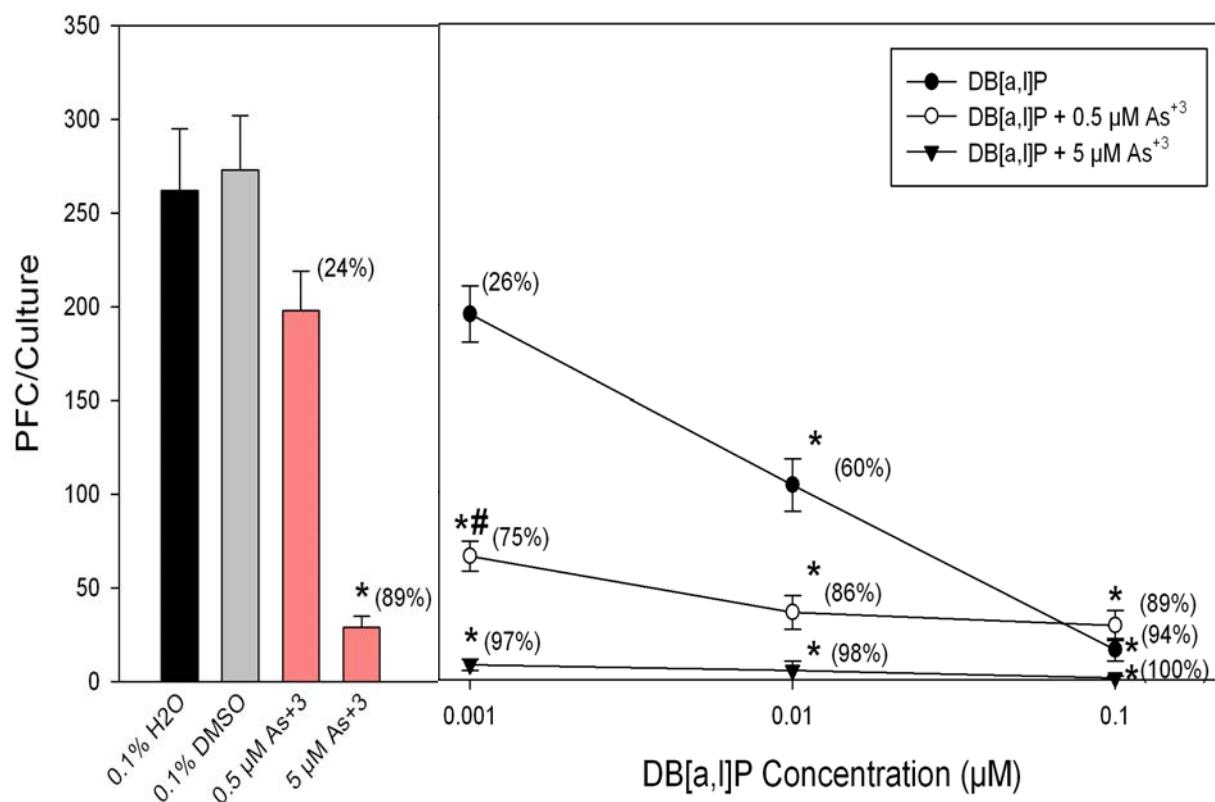
Spleen cells were immunized *in vitro* with SRBC and treated with DMSO or As<sup>3+</sup> and/or DMBA-diol. Immunotoxicity was determined by TDAR PFC assay as described in the Materials and Methods. No cytotoxicity compared to control was observed in these cultures compared to control culture. Data from a pool of three mouse spleens were assayed in quadruplicate and are shown as mean  $\pm$  S.E.M. Percentage indicates suppression relative to control (H<sub>2</sub>O for arsenic, DMSO for PAHs). \* indicates a statistically significant difference compared with vehicle control ( $p < 0.05$ ). # indicates a statistically significant effect of arsenite when added to PAHs compared to the PAH alone ( $p < 0.05$ ). Numbers in parenthesis indicate % suppression compared to vehicle control. Data are representative of two experiments.

***Immunosuppressive Effect of Co-Exposure to As<sup>3+</sup> and DB[a,l]P on C57Bl/6J mice***

***Splenocytes TDAR.***

In order to examine the co-effect of arsenite and DB[a,l]P, sodium arsenite at doses of 0.5  $\mu$ M and 5  $\mu$ M was chosen to combine with series doses of DB[a,l]P, ranging from 0.001  $\mu$ M to 0.1  $\mu$ M. Spleen cells were treated and immunized with SRBC at the same time. The TADR PFC assay was used to measure immunosuppression. As shown in Figure 11, DB[a,l]P produced a dose-dependent immunosuppression of the TDAR PFC response. Adding 0.5  $\mu$ M of sodium arsenite to the cell cultures greatly increased immunosuppression compared to DB[a,l]P by itself (0.001  $\mu$ M).



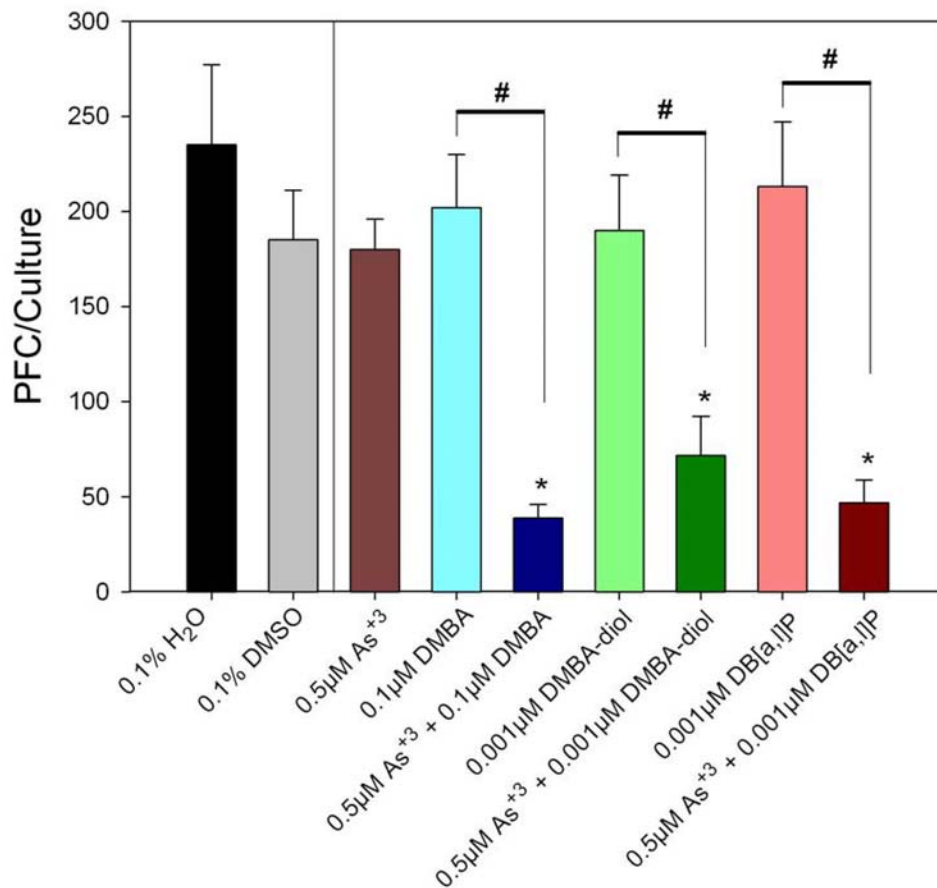


**Figure 11. Immunosuppressive Effect of Combining  $As^{3+}$  and DB[a,l]P on C57Bl/6J mice Splenocytes TDAR.**

Spleen cells were immunized *in vitro* with SRBC and treated with DMSO or  $As^{3+}$  and/or DB[a,l]P. Immunotoxicity was determined by TDAR PFC assay as described in the Materials and Methods. No cytotoxicity was observed in these cultures compared to control. Data from a pool of three mouse were assayed in quadruplicate and are shown as mean  $\pm$  S.E.M. \* indicates a statistically significant difference compared with vehicle control ( $p < 0.05$ ). # indicates a statistically significant effect of arsenite when added to PAHs compared to the PAH alone ( $p < 0.05$ ). Numbers in parenthesis indicate % suppression compared to vehicle control. Data are representative of two experiments.

***Synergistic Effect of Low Dose Arsenic and DMBA, DMBA-diol, and DB[a,l]P on the Spleen Cell TDAR in C57Bl/6J mice.***

The immunotoxicity of PAH and arsenic co-exposures were evaluated by treating C57BL/6J mice spleen cells with 0.5  $\mu\text{M}$  sodium arsenite with or without 0.1  $\mu\text{M}$  DMBA, 0.001  $\mu\text{M}$  DMBA-diol, and 0.001  $\mu\text{M}$  DB[a,l]P (Figure 12). These doses were chosen based upon results obtained in previous experiments that established a no-effect or low-effect level of exposure for these agents. As shown in Figure 11, 0.1  $\mu\text{M}$  DMBA, 0.001  $\mu\text{M}$  DMBA-diol, and 0.001  $\mu\text{M}$  DB[a,l]P did not produce significant suppression by themselves; however, by adding 0.5  $\mu\text{M}$  of sodium arsenite to the cell cultures, immunosuppression by each of these agents was greatly increased relative to the modest effects of arsenite alone. Because the individual agents at the concentrations employed did not produce significant immunosuppression on their own, these findings demonstrate the synergy between co-treatment of  $\text{As}^{3+}$  and DMBA, DB[a,l]P in suppression of the TDAR, a result that was also seen with BaP and its metabolites (below).

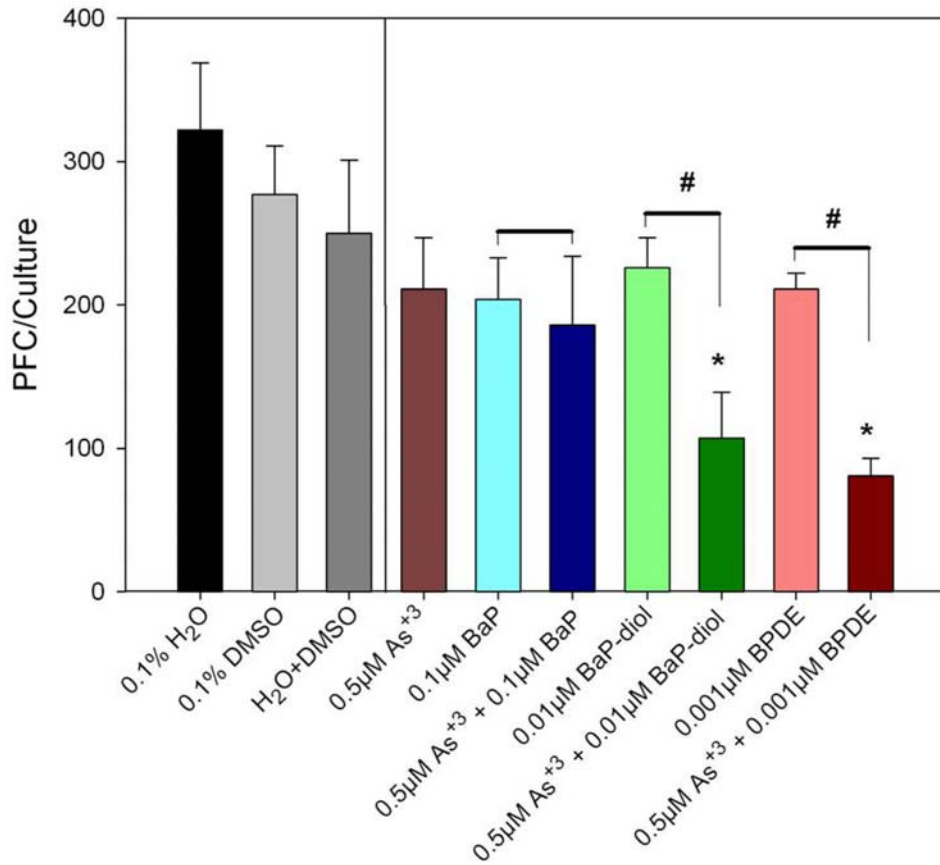


**Figure 12. Synergistic Effect of Low Dose Arsenic and DMBA, DMBA-diol, and DB[a,l]P on the Spleen Cell TDAR in C57Bl/6J mice.**

Spleen cells were immunized *in vitro* and treated with DMSO or H<sub>2</sub>O, DMBA, DMBA-diol or DB[a,l]P with or without As<sup>3+</sup>. Immunotoxicity was determined by TDAR PFC assay as described in Materials and Methods. No cytotoxicity was observed in these cultures compared to control. Data from a pool of three mouse spleens that were assayed in quadruplicate are shown as mean ± S.E.M. \* indicates a statistically significant difference compared with vehicle control (p < 0.05). # indicates a statistically significant effect of arsenite when added to PAHs compared to the PAH alone (p < 0.05). Data are representative of two experiments.

***Synergistic Effect of Low Dose Arsenic and BaP, BaP-diol and BPDE on the Spleen Cell TDAR in C57BL/6J mice.***

To evaluate effect of co-exposure of BaP and arsenic, we chose non-cytotoxic doses of BaP, BP-diol, and BPDE to combine with sodium arsenite to determine effects on the TDAR. We found that spleen cell cultures co-treated with low doses of sodium arsenite and BaP-diol as well as those treated with sodium arsenite and BPDE demonstrated synergistic immunosuppressive effects (Figure 13). 0.01  $\mu\text{M}$  BaP-diol and 0.001  $\mu\text{M}$  BPDE did not produce significantly suppression by themselves; however, when co-treated with 0.5  $\mu\text{M}$  of sodium arsenite to the culture media, we found significant immunosuppression. Combined with the results shown in Figure 11, these results demonstrate a synergistic interaction between PAHs and  $\text{As}^{3+}$ .



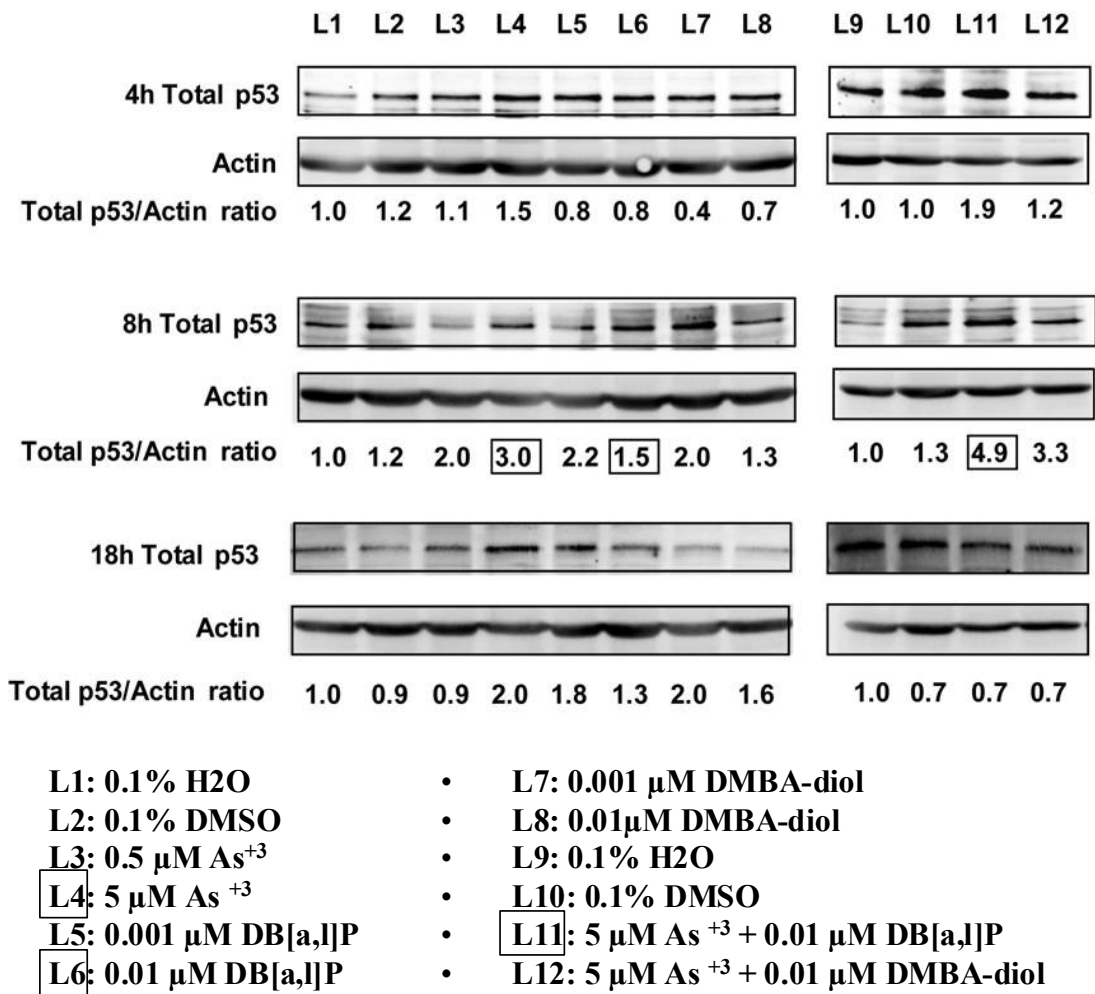
**Figure 13. Synergistic Effect of Low Dose Arsenic and BaP-diol and BPDE on the Spleen Cell TDAR in C57BL/6J mice.**

Spleen cells were immunized *in vitro* with SRBC and treated with DMSO, As<sup>3+</sup>, and/or BaP, BaP-diol and BPDE at the same time. The number of antibody producing spleen cells was determined by a modified Jerne and Nordin PFC assay as described in the Materials and Methods. Data from a pool of three mouse spleens were assayed in quadruplicate, and are shown as mean ± S.E.M. No cytotoxicity was observed in these cultures compared to control. \* indicates a statistically significant difference compared with vehicle control (p < 0.05). # indicates a statistically significant effect of arsenite when added to PAHs compared to the PAH alone (p < 0.05). Data are representative of two experiments.

## SPECIFIC AIM 2

### *As<sup>3+</sup> and PAHs alone and in combination cause p53 up-regulation in WT mice at 8 hrs.*

Previous studies in our laboratory have shown that DMBA triggers a significant p53 up-regulation in WT mice in a dose-dependent manner (Gao et al., 2008). In the current study, as shown in Figure 14, WT mice spleen cells were treated with sodium arsenite and/or DB[a,l]P for 4h, 8h and 18h. Western blot analysis was performed to examine total p53 protein levels. At 4 hour, the p53 level was not significantly increased by any of the treatments. The highest increase of p53 protein expression was observed following 8 hours of exposure. At this time point, 5  $\mu$ M sodium arsenite treatment resulted in a 3-fold increase of p53 protein levels; 0.01  $\mu$ M DB[a,l]P induces 1.5 fold increase of p53 protein compared to DMSO control. Combining 5  $\mu$ M sodium arsenite and 0.01  $\mu$ M DB[a,l]P up-regulated p53 protein by about 5-fold at 8 hour time point. Upon 18 hour treatment, p53 total protein level was decreased compare to 8 hour treatment. Taken together, Western blot results showed that p53 protein was up-regulated in a dose- and time-dependent manner following As<sup>3+</sup> and PAH treatments of murine spleen cells.



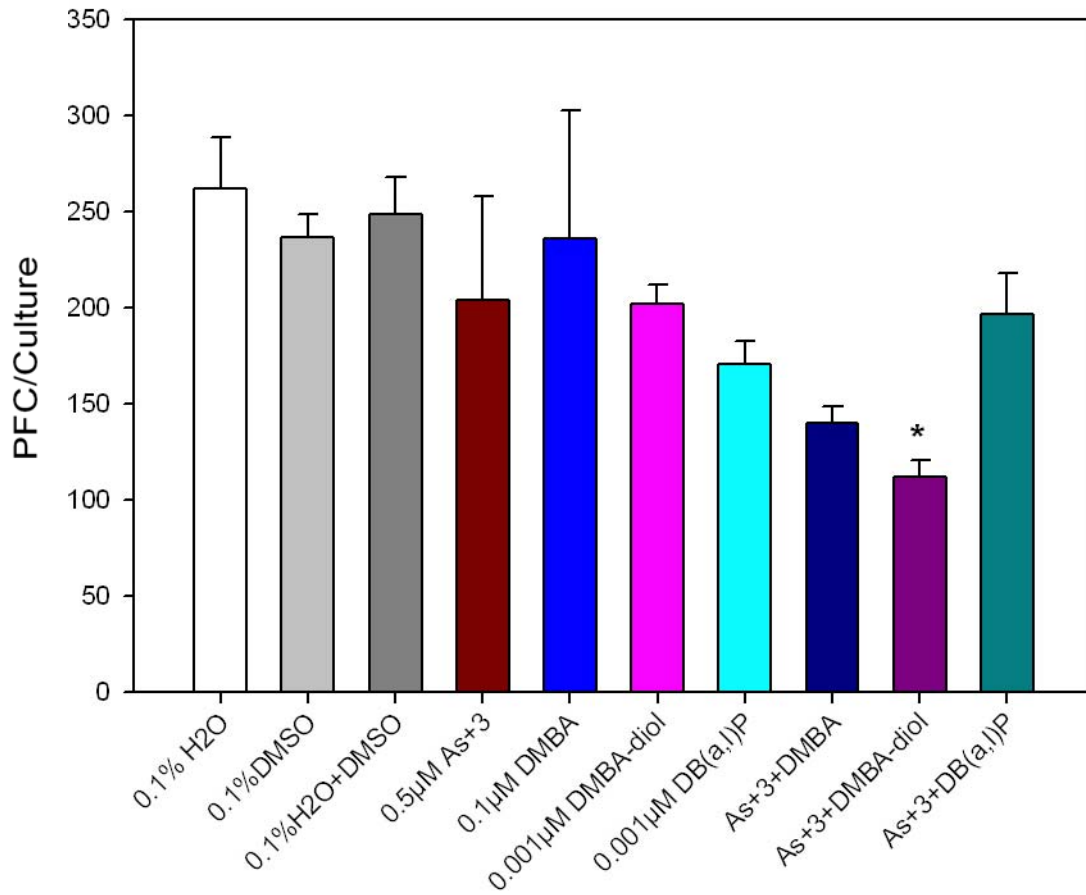
**Figure 14. Arsenic and PAHs Cause Total p53 Up-regulation in WT mice at 8 hour.**

Male WT mice spleen cells were collected as described in Material and Method, treated with DMSO,  $\text{As}^{3+}$  and/or DB[a,l]P for 4 h, 8 h and 18h. Following treatment, cells were lysed, and total protein was resolved by SDS-PAGE. Total p53 activity was determined by immunoblot with p53 1C12 antibody. The normalized protein levels were indicated as ratio at the bottom of each blot by densitometry analysis using  $\beta$ -actin as loading control.

### ***PAH-induced Immunosuppression was Partly Protected in p53-null Mice Spleen Cells.***

Previous studies in our laboratory showed that p53 knockout mice were protected from DMBA-induced immunotoxicity measured using an *ex vivo* spleen cell TDAR PFC (Gao et al., 2007). To determine if p53 is required for the arsenic/PAHs immunotoxicity in the current *in vitro* system, we immunized p53-null mice with sheep red blood cell, treated with the combination of 0.5  $\mu\text{M}$  of sodium arsenite and low dose of DMBA, DMBA-diol and DB[a,l]P. TDAR PFC assay was conducted to evaluate IgM antibody production as shown in Figure 15. In Table 1, we calculated the actual percentage of the TDAR suppression using a low dose (.001  $\mu\text{M}$ ) of DMBA-diol  $\pm$   $\text{As}^{3+}$  (.5  $\mu\text{M}$ ) and a low dose (.001  $\mu\text{M}$ ) of DB[a,l]P  $\pm$   $\text{As}^{3+}$  (.5  $\mu\text{M}$ ), and compared the data from p53-null mice to WT mice. We found that spleen cells from p53 null mice exposed to DMBA-diol +  $\text{As}^{3+}$  were significantly protected from immunosuppression as compared to the WT mice. As shown, we found that p53-null mice were protected from the  $\text{As}^{3+}$  + DMBA-diol (96% suppression in WT mice and only 46% suppression in p53 null mice), as well as the  $\text{As}^{3+}$  + DB[a,l]P immunosuppression (75% suppression in the WT mice and only 21% suppression in the p53 null mice). Interestingly, immunotoxicity produced by the single low dose exposures to DMBA-diol,  $\text{As}^{3+}$ , or DB[a,l]P were less protected in the p53 null mice.





**Figure 15. PAH-induced Immunosuppression was Partly Protected in p53-null mice Spleen Cells.**

Spleen cells from three p53-null mice were pooled, immunized *in vitro* and treated with DMSO, As<sup>3+</sup>, and/or DMBA, DMBA-diol and DB[a,l]P. The number of antibody producing spleen cells was determined by a TDAR PFC assay as described in Materials and Methods. Data from a pool of three mice spleens were assayed in quadruplicate. Data are shown as mean  $\pm$  S.E.M. No cytotoxicity was observed in these cultures compared to control. \* indicates a statistically significant difference compared with 0.1% H<sub>2</sub>O+ 0.1% DMSO ( $p < 0.05$ ). Data are representative of two experiments.

**Table 1. PercentSuppressions of TDAR by PAHs ± As<sup>3+</sup> in WT vs. p53-null mice.**

Numbers are representatives of the TDAR suppression percentage compared to vehicle controls.

Data for WT for the treatments 0.001 μM DMBA-diol, 0.5 μM As<sup>3+</sup> and DMBA-diol + As<sup>3+</sup> in

WT are from Figure 10, Data for WT for the treatments 0.001 μM DB[a,l]P, 0.5 μM As<sup>3+</sup> and

DB[a,l]P + As<sup>3+</sup> in WT are from Figure 11. p53-null data are from Figure 15.

	0.001 μM DMBA-diol	0.5 μM As <sup>3+</sup>	DMBA-diol + As <sup>3+</sup>	0.001 μM DB[a,l]P	0.5 μM As <sup>3+</sup>	DB[a,l]P + As <sup>3+</sup>
<b>C57BL/6J WT</b>	36%	36%	96%	26%	24%	75%
<b>p53-null</b>	23%	23%	46%	30%	23%	21%

## DISCUSSION

Environmental exposure of humans to PAHs and arsenic is a concern from a global health perspective. EPA has classified both classes of chemical agents as important carcinogenic and immunotoxic chemicals. The present studies were designed to characterize the immunosuppression of PAHs and arsenic in spleen cells from C57BL/6J mice using a T-dependent antibody response, and to study possible mechanisms of their interaction. The murine immune system was proved previously to be an excellent predictor of human immunosuppression produced by environmental chemicals (Vos and Van, 1998).

PAH immunosuppression has been studied for decades in animal models (Schnizlein et al., 1987; Burchiel et al., 1990; Gao et al., 2005) and in cell lines (Davila et al., 1996). BaP and DMBA are prototypical PAHs that are well characterized immunosuppressants. In the current study, we investigated the TDAR *in vitro* in C57BL/6J mice spleen cells exposed to BaP, DMBA and their metabolites, and compared their different potencies. The TDAR is a sensitive and commonly utilized assay for assessment of immunosuppression (White et al., 2010). A dose-dependent decrease in TDAR was observed in the presence of the T-dependent antigen, sheep red blood cells, for each investigated PAH. Metabolites exhibited much higher potency than parent compounds. This is likely to be explained by previous studies which shows that DMBA bioactivation requires CYP and mEH to be genotoxic and immunosuppressive (Gao et al., 2005; Gao et al., 2007; Gao et al., 2008). Uno et al. have also previously shown that CYP1B1 is required for BaP metabolism and immunotoxicity (Uno et al., 2006).

DB[a,l]P is an important environmental PAH, which has been identified along with other PAHs, in ambient particulate matter (PM) samples and other sources (Bergvall et al., 2007).

DB[a,l]P has been previously studied as one of most potent carcinogens evaluated in the mouse

model (Castro et al., 2008). Treatment of pregnant mice with DB[a,l]P resulted in the formation of a highly progressive T-cell lymphoma in their offspring. Mice that survived to 10 months of age developed lung tumors. Previous studies indicated that DB[a,l]P shared some chemical and biological characteristics similar to DMBA (Melendez-Colon et al., 1999). DB[a,l]P is bioactivated by CYP1B1 and leads to the formation of metabolite that interact with DNA, forming an AP site and causing DNA damage (Castro et al., 2008; Busters et al., 2002). To our knowledge, no previous study has examined the immunotoxicity of DB[a,l]P. In the present studies, we hypothesized that DB[a,l]P would be a potent immunosuppressant of the TDAR based upon its known genotoxicity. In agreement with this hypothesis, we found that DB[a,l]P produced a dose-dependent immunosuppression of the TDAR in the *in vitro* murine spleen cell system. In addition, we found that DB[a,l]P is about 10-fold more potent than DMBA. The lowest concentration of DB[a,l]P that produced a statistically suppression of TDAR is 0.001  $\mu\text{M}$ . Thus, our studies showed that DB[a,l]P is a potent immunosuppressant.

Turning our attention to the other environmental chemical examined in these studies, we know that arsenic is a widely distributed contaminant in the environment. Immune suppression by arsenic has been observed in previous studies *in vitro* and *in vivo* in animal models and in humans living in arsenic contaminated areas. Early studies in the mouse model showed that a 200 mg/kg intratracheal exposure of gallium arsenide impairs both humoral and cellular immune responses (Sikorski et al., 1989). Recent studies in our laboratory showed that 50  $\mu\text{g}/\text{m}^3$  and 1  $\text{mg}/\text{m}^3$  inhalation exposures of arsenic trioxide produced extremely suppression of the TDAR to sheep red blood cells (Burchiel et al., 2009). This indicates potential risk of lung diseases that might result from arsenic inhalation exposure. A study that investigated immune effects of arsenic in drinking water in an exposed population of children living in central Mexico showed

that T cell activation was suppressed (Soto-Peña et al., 2006). A more recent study in individuals living in a arsenic contaminated area in India, found a significant decrease in cytokines produced by T cells and a marked dose-dependent suppression of Concanavalin A (Con A) induced proliferation responses in exposed individuals (Biswas et al., 2008).

In the present study, we characterized TDAR immunosuppression in mouse splenocytes exposed to sodium arsenite ( $As^{3+}$ ) or sodium arsenate ( $As^{5+}$ ). These are two inorganic forms of arsenic found in the environment. We found arsenite was 10-fold more potent in suppressing TDAR than arsenate in our system. This is consistent with previous studies, showing that pentavalent arsenic is more potent than trivalent arsenic in suppressing the function of pulmonary alveolar macrophages (Lantz et al., 1994). In addition, previous studies have shown that  $As^{3+}$  induces more ROS production and DNA damage than  $As^{5+}$  (Ding et al., 2009). These might be important mechanisms to explain the observation in our study that  $As^{3+}$  was more potent in inducing immunosuppression than  $As^{5+}$  in our system.

Our studies also investigated the immunosuppressive effects produced of arsenic and PAH co-exposure in normal C57/BL6J mouse splenocytes. Our results indicated that at low doses,  $As^{3+}$  potentiated the immunosuppression produced by PAHs, specifically DMBA, DMBA-diol, DB[a,l]P, BaP-diol and BPDE. A concentration of  $0.5\mu M$  of  $As^{3+}$  by itself did not cause significant immune suppression, whereas combined treatments of low doses arsenic and PAHs significantly potentiated immunosuppression. These studies indicate to us that low levels of arsenic and PAH co-exposure may have synergistic immunosuppressive effects. This is the first report that describes the co-effect of arsenic and PAHs in immunosuppression. The  $0.5\mu M$  level (65 ppb) of arsenic used in this study to show synergism with PAHs was only modestly higher than the current EPA limit for arsenic in drinking water in the United States 10 ppb. Many

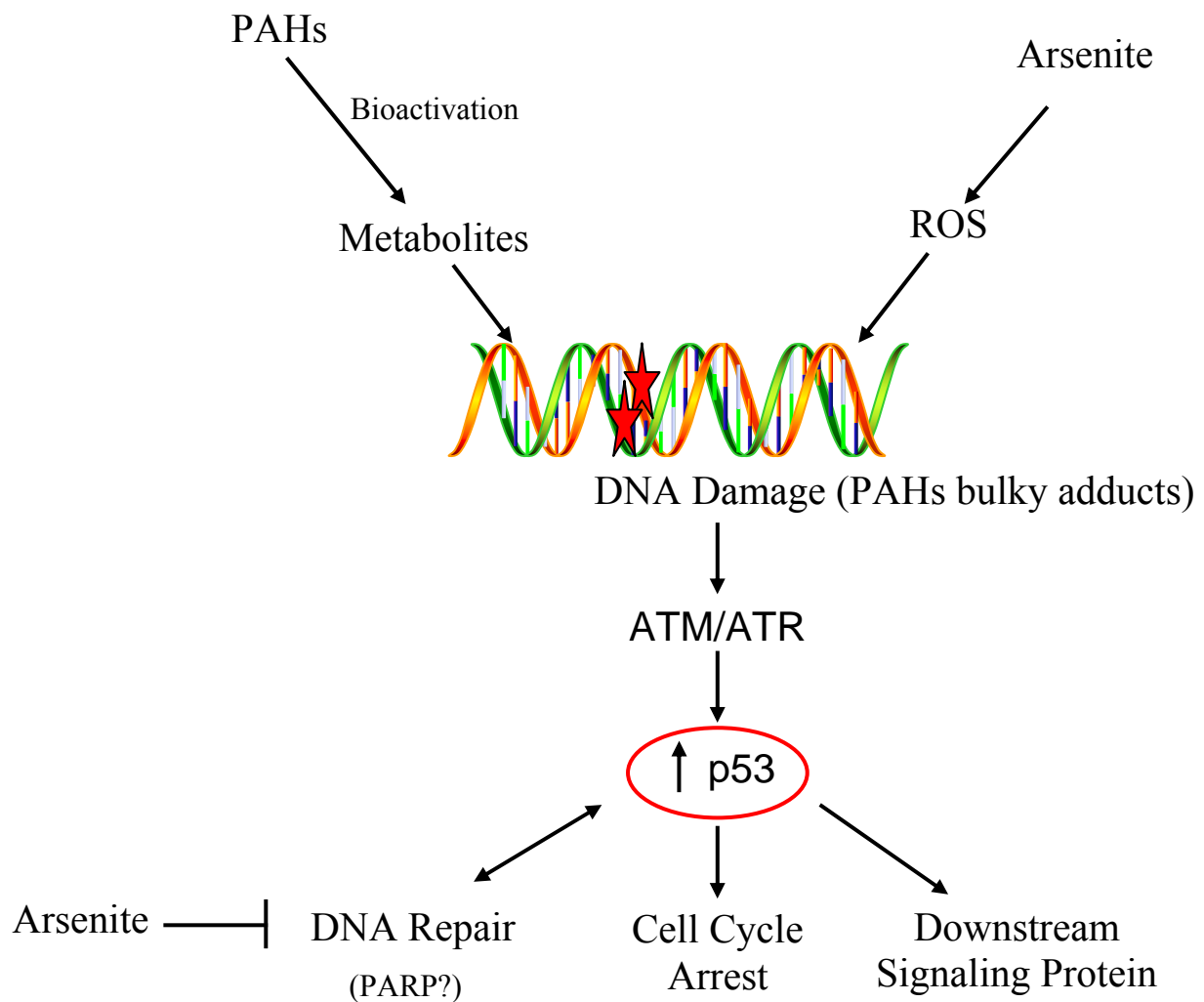
world populations are exposed to levels of arsenic in the range of 100-1000 ppb. Therefore these studies suggest that further environmental arsenic toxicity assessments should be performed and evaluated in human populations.

Regarding potential mechanisms whereby PAHs may interact with  $As^{3+}$  to produce immunosuppression, we hypothesized that there is a relationship between the amount of genotoxicity produced by xenobiotics and their resultant immunotoxicity. Luster et al have shown that many genotoxic agents are immunosuppressant (Luster et al., 1992). Recent studies have also shown that arsenic greatly potentiates BaP genotoxicity (Maier et al., 2002). DNA adducts levels in mouse skin and lung produced by BaP were found to be increased 8-fold by arsenic (Evans et al., 2004). These studies suggest that arsenic and BaP interacted *in vivo* and *in vitro*. While the mechanism of arsenic and PAH interaction remains unexplained, these studies provide important rationale for present study on low doses of arsenic and PAH synergistic immunosuppression of the TDAR. We believe that genotoxicity is the major pathway for arsenic and PAH induced immunotoxicity. As shown in Figure 16, we hypothesize that PAH metabolites that are formed via P450 and epoxide hydrolase activation form bulky adducts that are genotoxic leading to DNA strand breaks, mutations and cancer. These adducts are sensed by ATM/ATR, leading to p53 activation and immunosuppression. This conclusion is based upon a recent series of studies in our laboratory, showing that DMBA, in the presence of mEH and CYP1B1 produces immunotoxicity via a genotoxic mechanism (Gao et al 2008). Gao et al also found that p53 was increased by DMBA via ATM/ATR activation, and presumably cell cycle inhibition which is associated with immunosuppression.

We believe that  $As^{3+}$  may interfere with DNA repair by replacing Zn in Zn-finger proteins leading to inhibition of protein activity. A current hypothesis under evaluation in our

laboratories (collaboration with Liu and Hudson labs) is that one such Zn-fingered protein that may be inhibited by  $As^{3+}$  is Poly (ADP-ribose) Polymerase-1 (PARP). The role of PARP in the repair of DNA bulky adducts such as PAHs has not been well-established. However, a recent study demonstrated that a low concentration of sodium arsenite inhibited and interfered with DNA repair machinery in human keratinocytes (Qin et al., 2008; Ding et al., 2009). Thus, we believe that by interfering with the DNA repair system, arsenic might potentiate the immunotoxicity of PAHs by increasing the amount of unrepaired adducts leading to enhanced genotoxicity (shown in Figure 16). We also cannot rule out a role for oxidative stress-associated pathways that may play a role in the formation oxidative adducts, such as 8-OHdG, that mediate non-genotoxic signaling pathways.

In support of a genotoxic mechanisms of action for the combined effects of PAHs and  $As^{3+}$ , we found that p53 total protein levels were increased in mouse spleen cells treated with  $As^{3+}$  and PAHs alone or in combination. The fact that p53 was increased for both  $As^{3+}$  and PAHs provides initial evidence for this hypothesis. Co-treatment of spleen cells with  $As^{3+}$  and PAH induced a significantly greater up-regulation of p53 compared to  $As^{3+}$  or PAH given alone. Previous studies have shown that p53 is induced following arsenic exposure. Arsenic trioxide was shown to induce p53 in a dose-dependent manner in human leukemia cell lines (Yedjou et al., 2009). Chronic exposure of low level arsenite in human keratinocytes exhibited poly(ADP-ribosyl)ation of p53 which interferes with p53 activation (Komissarova et al., 2010). Thus the findings reported in the present studies are consistent with literature reports on the induction of p53 by  $As^{3+}$ .



**Figure 16. Schematic for Genotoxic Mechanisms of Arsenite and PAHs Co-exposure Induced Immunotoxicity.**



In further support of our genotoxicity and p53 hypothesis for PAH and As<sup>3+</sup> interactions, we found that p53-null mice were mostly protected from immunosuppression produced by arsenic and PAH co-treatment. This result is consistent with previous studies showing that p53 is required for DMBA-immunotoxicity (Gao et al., 2008). Further *in vivo* studies are needed in p53-null mice. However, these initial results are consistent with our current thinking that p53 is a key regulator of immunosuppression produced by arsenic and PAH co-exposures in WT mice.

While p53 and genotoxicity appear to play a central role in As<sup>3+</sup> and PAH-induced immunosuppression, we also have some evidence for p53-independent pathways of PAH and As<sup>3+</sup>, based on the findings that a small percentage of TDAR immunosuppression is seen in p53 null mice. Therefore, we have considered other mechanisms for PAH and As<sup>3+</sup> that may involve non-genotoxic pathways (Burchiel and Luster, 2001). PAHs are substrates of peroxisomes and produce ROS generative quinone, which cause oxidative stress in the cells (Burchiel and Luster, 2001). PAHs are also able to induce Ca<sup>2+</sup> elevation and trigger a series of downstream signaling pathways that may lead to anergy (tolerance) (Burchiel and Luster, 2001). Recent research showed a synthetic bioactive peptide, CKS-17, potently suppresses Th1 type immune response by activating several intracellular signaling molecules, such as elevating intracellular cyclic adenosine monophosphate (cAMP) levels, and inducing phosphorylation of extracellular signal-regulated kinase (ERK) 1 and 2 (Haraguchi et al., 2008). A recent study showed that arsenite induced cell cycle arrest and apoptosis in p53-proficient and p53-deficient mouse embryonic fibroblasts (MEFs) through different mechanisms (Yu et al., 2008). In p53<sup>+/+</sup> MEFs, arsenite induced alteration of genes responsible for DNA damage and cell cycle arrest, whereas in p53<sup>-/-</sup> cells, arsenite induced significant up-regulation of pro-apoptotic genes and down-regulation of

immune modulation (Yu et al., 2008). These studies might help us better understand that while p53 plays an important part of As<sup>3+</sup> and PAHs induced immunosuppression, there might be more than one mechanism responsible for xenobiotic-induced immunosuppressions.

In summary, this study utilized the TDAR to characterize the dose-dependent immunosuppression of DMBA, DB[a,l]P, BaP, their metabolites, alone or in combination with As<sup>3+</sup>. Metabolites of PAHs were found to be more potent than parent compound. As<sup>3+</sup> was 10-fold more suppressive than As<sup>5+</sup>. In addition, As<sup>3+</sup> at a low dose of 0.5 µM greatly potentiated low dose PAH immunosuppression. p53 total protein expression was found to be significantly increased by treating mouse splenocytes with these arsenic and PAHs alone or in combination. In addition, spleen cells from p53-null mice were found in these studies to be partly protected from As<sup>3+</sup> and PAH immunosuppression.

Previous reports have established a positive correlation between immunosuppression and carcinogenicity produced by genotoxic chemicals (Luster et al., 1992, 1993). However, our data suggest that the important pathway associated with genotoxicity-induced immunosuppression is p53. Because there is an apparent correlation between carcinogenesis and immunosuppression produced by genotoxic chemicals (Luster et al., 1992), the question may arise as to the role of immunosuppression in tumor formation *in vivo*. The immune system plays an important role in the prevention of cancers induced by environmental chemicals, including PAHs such as 3-methylcholanthrene (Schreiber and Pollack, 2009). A competent immune response is required to eliminate tumor cells by recognition of the antigen expressed by tumor cells. Recent clinical and experimental studies further support this conclusion by showing that immunosuppressants, such as cyclosporine (CsA), increase the incidence of skin cancer in organ transplant recipients (Euvrard et al., 2004). Thus, our studies suggest that there may be common

mechanisms of tumor induction and immunosuppression associated with the genotoxic actions of PAHs. The genotoxicity of PAHs may be further exacerbated by co-treatments with arsenic.

While the present research provides insights into arsenic and PAHs co-exposure induced immunosuppression, there are some limitations in this study. First, we know that an *in vitro* system does not account for all of the *in vivo* actions of PAHs and arsenic in either animals or humans. In the future it will be interesting to investigate the immunosuppressive mechanism(s) produced by arsenic and PAHs using *in vivo* models and to evaluate their relevance to potential human exposures. Additional studies examining potential mechanisms of As<sup>3+</sup> and PAHs synergistic immunosuppression will be needed in the future, including *in vivo* modeling of p53-dependent and p53-independent pathways. These studies will help us better understand the potential environmental risks that might be associated with human exposures and associated immune suppression.

## LIST OF ABBRVIATIONS

2-ME	2-Mercaptoethanol
AAALAC	Association for assessment and accreditation of laboratory animal care
Ab	Antibody
ATM	Ataxia Telangiectasia Mutated protein
ATR	Ataxia Telangiectasia- and Rad3-related protein
ASTDR	Agency for Toxic Substances and Disease Registry
BaP	Benzo[a]pyrene
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Con A	Concanavalin A
CsA	Cyclosporine
CYP	Cytochrome P450
CYP 1A1	Cytochrome P450 1A1
CYP 1B1	Cytochrome P450 1B1
DB[a,l]P	Dibenzo[a,l]pyrene
DMA	Dimethylarsenic acid
DMBA	7,12-Dimethylbenz[a]-anthracene
DMBA-diol	7,12-Dimethylbenz[a]-anthracene-3,4-dihydrodiol
DPBS	Dulbecco's phosphate buffered saline

DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetate acid
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GSH	Glutathione
HBSS	Hanks Balanced Salt Solution
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
MEFs	Mouse embryonic fibroblasts
mEH	Microsomal epoxide hydrolase
MMA	Monomethylarsenic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
PAHs	Polycyclic Aromatic Hydrocarbons
PARP	Poly (ADP-ribose) Polymerase-1
PFC	Plaque-forming cell assay
PM	Particulate matter
PMSF	Phenylmethylsulfonyl fluoride
RPMI	Rowell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

SRBC	Sheep red blood cell
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline with Tween 20
TMAO	Trimethylarsine oxide
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
WT	Wild-type

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