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Polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BP) are ubiquitous environmental carcinogenic contaminants exerting deleterious effects toward cells acting in the immune defense such as monocytic cells. To investigate the cellular basis involved, we have examined the consequences of PAH exposure on macrophagic differentiation of human blood monocytes. Treatment by BP markedly inhibited the formation of adherent macrophagic cells deriving from monocytes upon the action of either GM-CSF or M-CSF. Moreover, it reduced expression of macrophagic phenotypic markers such as CD68 and CD14 in GM-CSF-treated monocytic cells, without altering cell viability or inducing an apoptotic process. Exposure to BP also strongly altered functional properties characterizing macrophagic cells such as endocytosis, phagocytosis, LPS-triggered production of TNF-α and stimulation of allogeneic lymphocyte proliferation. Moreover, formation of adherent macrophagic cells was decreased in response to PAHs distinct from BP such as dimethylbenz(a)anthracene and 3-methylcholanthrene, which interact, like BP, with the arylhydrocarbon receptor (AhR) known to mediate many PAH effects. In contrast, benzo(e)pyrene, a PAH not activating AhR, had no effect. In addition, AhR was demonstrated to be present and functional in cultured monocytic cells, and the use of its antagonist α-naphthoflavone counteracted inhibitory effects of BP toward macrophagic differentiation. Overall, these data demonstrate that exposure to PAHs inhibits functional in vitro differentiation of blood monocytes into macrophages, likely through an AhR-dependent mechanism. Such an effect may contribute to the immunotoxicity of these environmental carcinogens owing to the crucial role played by macrophages in the immune defense. The Journal of Immunology, 2003, 170: 2374–2381.

P olycyclic aromatic hydrocarbons (PAHs) represent an important class of widely distributed environmental contaminants. They are usually formed through the combustion of fossil fuel and the burning of various substances and are found in significant amounts in automobile exhaust, cigarette smoke, various foods, and industrial waste by-products. They can exert major toxic effects, including development of cancers in various tissues, cardiovascular diseases, loss of fertility, and immunosuppression (2–5). Many of these adverse effects are thought to be linked to the cytosolic arylhydrocarbon receptor (AhR), a ligand-dependent basic helix-loop-helix transcription factor to which PAHs bind, thereby triggering translocation of the AhR into the nucleus, association with the AhR nuclear translocator (ARNT), and ultimately interaction with xenobiotic responsive elements found in the 5′ flanking regions of responsive genes (6). In this way, AhR is notably implicated in up-regulation of drug-metabolizing enzymes such as cytochromes P450 (CYPs), especially CYP1A1 (7), which metabolize PAHs into reactive intermediates that are capable of interacting with DNA and most likely account for mutagenic properties of these environmental contaminants (8).

Immunosuppression due to PAHs, which may indirectly contribute to their carcinogenic properties, also involves (at least in part) AhR, because the AhR antagonist α-naphthoflavone counteracts some adverse effects of PAHs toward immune cells (4, 9). However, cellular and molecular mechanisms involved in PAH-related immunotoxicity remain incompletely understood, especially for human cells, because most published studies have been performed on mice (9–11). Lymphocytes likely constitute important targets. Indeed, potent immunosuppressive PAHs such as benzo(a)pyrene (BP) and dimethylbenz(a)anthracene (DMBA) inhibit murine T and B cell proliferation and alter T cell-related cytokine production and B cell-mediated Ab production (11–13). They also suppress mitogenesis of human T lymphocytes (14) and alter B cell lymphopoiesis through triggering pre-B lymphocyte apoptosis (15).

Besides lymphocytes, APCs such as macrophages and dendritic cells can also be affected by PAHs. Indeed, PAHs impair Ag presentation by mouse macrophages (16), alter T cell-macrophage interaction (17), and suppress phagocytic activity of peritoneal macrophages (18). BP also reduced esterase-positive macrophagic cell population in mouse spleen (19). In fact, murine splenic macrophages, which have been demonstrated to metabolize PAHs (20), are considered the cell types targeted by BP among the different splenic cell populations and are responsible for PAH-related suppression of splenic humoral immune response (21). Moreover, it is noteworthy that cigarette smoke condensates, whose major components consist of PAHs, markedly down-regulate functional

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capacities of macrophages (22). More specialized APCs, i.e., Langerhans cells, have also been found to be altered after topical application of DMBA (23). Interestingly, PAHs have been recently demonstrated to strongly impair functional differentiation and maturation of human monocytes into dendritic cells (24). This indicates that differentiation pathways of monocytes may be compromised by PAHs. Owing to the fact that macrophages are likely major targets of PAHs as reported above, it is tempting to speculate that PAHs may affect not only dendritic cell differentiation, but also macrophage generation from monocytes. To test such a hypothesis, in the present study we have examined the effects of PAH exposure on functional differentiation of peripheral blood monocytes into macrophages upon the action of cytokines such as GM-CSF and M-CSF. Our data indicate that PAHs inhibit generation of functional macrophagic cells from human monocytes likely through, at least in part, an AhR-related mechanism. Such an altered macrophagic differentiation may significantly contribute to PAH-related immunotoxicity, owing to the crucial role played by macrophages in the immune response, and supports the conclusion that monocyte differentiation pathways represent major cellular events affected by PAHs.

Materials and Methods

Chemicals and reagents

BP, DMBA, 3-methylcholanthrene (MC), benzo(e)pyrene (BeP), α-naphthoflavone, LPS, PMA, and FITC-dextran (Mr, 40,000 kDa) were provided by Sigma-Aldrich (St. Louis, MO). Dihydrodorhamadine 123 and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). Chemicals were commonly used as stock solutions in DMSO. Final concentrations of solvent in culture medium did not exceed 0.2% (v/v); control cultures were commonly used as stock solutions in DMSO. Final concentrations of solvent in culture medium did not exceed 0.2% (v/v); control cultures received the same dose of solvent as for treated counterparts. [3H]Thymidine (sp. act., 5 Ci/mmole) was obtained from Amersham (Les Ulis, France). Fluorescent latex microspheres were purchased from Polysciences (War- rington, PA). The tetrazolium salt 4-[(3-[4-isopolyphenyl]-2-[4-metaphenyl]-2H-5tetrazoie]-1,3-benzenesulphonate (WST-1) was provided by Roche Diagnostics (Meylan, France). Human GM-CSF (sp. act., 1–10 8 U/mg) was obtained from Promocell (Heidelberg, Germany).

Preparation and culture of human monocytes

PBMCs were obtained from blood buffy coats of healthy donors through Ficoll gradient centrifugation and from cyathopheresis products of nonpatho- logical peripheral blood. Monocytes were then prepared by a 2-h adhesion step, which routinely obtained >90% of adherent CD14-positive cells as assessed by immunostaining. These monocytic cells were next cultured in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics, and 10% FCS in the presence of 800 U/ml GM-CSF or 50 U/ml M-CSF to get macrophages as previously reported (25).

Cellular viability determination

Cellular viability was determined by microscopic analysis of cellular ex- clusion of trypan blue dye and flow cytometric analysis of cellular pro- pidium iodide staining as previously reported (24).

Cellular adhesion assay

Adherent fraction of cultured monocytic cells was analyzed using the WST-1 assay in 96-well microplates. Briefly, after careful removal of non- adherent cells by gentle aspiration, adherent cells were washed and then incubated with 10 μl of WST-1. The yellow formazan product formed by cellular metabolism was determined in parallel from wells containing monocytic cells alone and was calculated as follows: mean fluorescence (mAb of interest) – mean fluorescence (control mAb).

Cytokine measurements

Levels of TNF-α in the supernatants of LPS-treated monocytic cultures were quantified using an ELISA kit obtained from BD Biosciences. Analyses were conducted according to the manufacturer’s instructions.

Endocytosis assay

Cultured monocytic cells were incubated with 1 mg/ml FITC-dextran for 60 min at 37°C. Cellular uptake of FITC-dextran was then monitored by flow cytometry. A negative control was performed in parallel by incubating cells with FITC-dextran at 4°C instead of 37°C.

Phagocytosis assay

Cultured monocytic cells were incubated with 15 μl of fluorescent latex microspheres for 30 min at 37°C. Cellular phagocytosis of latex beads was then monitored by flow cytometry. A negative control was performed in parallel by incubating cells with latex beads at 4°C instead of 37°C.

Mixed leukocyte reaction

Ability of cultured monocytic cells to stimulate allogeneic T cell prolifer- ation was determined using MLR as previously described (24). Briefly, 3.5 × 10 5 LPS-pretreated monocytic cells were cultured with 1.5 × 10 5 alllogenic T cells in round-bottom 96-well microplates. After 5 days of culture, cells were pulsed with 1 μCi of [3H]thymidine for 18 h. Incorpora- tion of the radionucleide into DNA was further measured by scintillation counting. Basal thymidine incorporation into monocytic cells was deter- mined in parallel from wells containing monocytic cells alone and was subtracted from thymidine counts observed in T cell-monocytic cell co- cultures. Results were expressed as radioactivity (cpm) per well.

Respiratory burst activity detection

Respiratory burst activity detection was performed using the reactive oxygen intermediate-sensitive probe dihydrodorhamadine 123 (26). Monocytic cells were first prestimulated for 45 min with 100 ng/ml PMA, a chemical commonly used to trigger oxidative burst activity in phagocytic cells (27). After washing, cells were incubated with 5 μg/ml dihydrodorhamadine 123 for 45 min at 37°C. Cellular fluorescence due to rhodamine 123, formed upon the action of reactive oxygen intermediates such as hydrogen peroxide, was then measured by flow cytometry. Data were expressed as fluo- rescence arbitrary units.

RNA isolation and RT-PCR analysis

Total RNA was isolated from cells using the guanidinium thiocyanate/ cesium chloride method of Chirgwin et al. (28). RT-PCR analysis of CYP1A1, AhR, ARNT, and β-actin mRNA expression was then performed as previously reported (24). The primers used for CYP1A1, AhR, and ARNT detection were exactly those used by Baron et al. (29) and Roberts et al. (30). β-Actin detection was performed as a loading control with the following primers: sense, 5′-GCCAGAAGCAGAAGGATG-3′, and anti- sense, 5′-GGCATCTCTTGCTCG-3′. PCR products were separated on 1.2% agarose gels and stained with ethidium bromide.
Statistical analysis
Data were analyzed with the nonparametric Wilcoxon’s test. The level of significance was *p* < 0.05.

Results

**PAHs alter the formation of adherent macrophagic cells in GM-CSF-treated monocyte cultures**

Blood monocytes cultured for 6 days in the presence of GM-CSF developed into adherent macrophagic cells displaying a “fried-egg”-like morphology, i.e., round cells with a large nucleus centered in the cytoplasm, as previously reported (25) (Fig. 1). In the presence of 10 μM BP throughout the culture, the formation of adherent macrophagic cells was markedly reduced, and most of the cells remained as nonadherent cells in the culture medium and exhibited a smaller size than their untreated counterparts (Fig. 1). However, BP treatment did not result in alteration of cell viability as assessed by determination of trypan blue exclusion (Fig. 2A) and by measurement of the percentages of propidium iodide-stained cells (Fig. 2B). In addition, the proportion of apoptotic cells remained low, i.e., below 10%, in both BP-treated and untreated monocytic cultures as assessed by Hoechst 33342 labeling of apoptotic nuclei (Fig. 2C).

The decrease in the formation of adherent macrophagic cells in response to BP was further quantified using the WST-1 assay. As shown in Fig. 3A, 10 μM BP reduced adherent monocytic cell number to ~25–30% of that found in untreated cultures. Such a decrease was similarly observed in BP-exposed monocytic cells maintained in culture for 4, 6, or 10 days. Shorter exposure to BP, i.e., < 4 days, was not tested since a 4- to 6-day culture period is usually required for getting macrophages from monocytes in response to GM-CSF (25). The action of BP was dose dependent: concentrations such as 10 and 1 μM maximally reduced adherent macrophagic cell number, whereas the addition of 0.5 μM BP resulted in only a partial effect, and lower concentrations such as 0.1 and 0.01 μM had no effect (Fig. 3B). Besides BP, other PAHs used at 10 μM, such as DMBA and MC, also decreased the formation of adherent macrophagic cells in GM-CSF-treated monocytic cultures (Fig. 3C). In contrast, 10 μM BeP failed to alter the adherent macrophagic population (Fig. 3C).

**PAHs alter expression of phenotypic surface markers in GM-CSF-treated monocytic cell cultures**

To investigate the effects of PAHs on expression of surface markers, we first analyzed levels of CD71 in PAH-treated and untreated monocytic cells cultured in the presence of GM-CSF for 6 days. Indeed, CD71, which corresponds to the transferrin receptor, is well known as a macrophagic differentiation marker (31, 32). In agreement with this point, monocytic cells, which initially did not express CD71, exhibited a marked expression of this surface marker after 6 days of culture, i.e., when they had acquired a macrophagic phenotype (Fig. 4). In the presence of PAHs such as BP and DMBA, however, CD71 expression remained very low (Fig. 4). In contrast, CD71 levels were unaffected by BeP (Fig. 4).

**Statistical analysis**

Data were analyzed with the nonparametric Wilcoxon’s test. The level of significance was *p* < 0.05.

**Results**

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**Statistical analysis**

Data were analyzed with the nonparametric Wilcoxon’s test. The level of significance was *p* < 0.05.
various BP concentrations (from 0.01 to 10 μM) in H9262 A cells (Fig. 6). This indicates that LPS-stimulated release of TNF-α by monocytes was found to markedly suppress cellular endocytosis of FITC-dextran when compared with untreated cells (Fig. 5A). Similarly, cellular phagocytosis of fluorescent microspheres was down-regulated in response to BP (Fig. 5B). Addition of BP during macrophagic differentiation also markedly diminished LPS-mediated secretion of TNF-α by monocytes as demonstrated by ELISA measurements of TNF-α levels in culture supernatants (Fig. 6A). This indicates that LPS-stimulated release of TNF-α was likely impaired in PAH-treated cells. BP exposure also strongly inhibited the ability of cultured monocytes to induce proliferation of allogeneic T lymphocytes. Indeed, unlike their untreated counterparts, BP-exposed monocytes failed to stimulate DNA synthesis in allogeneic T lymphocytes (Fig. 6B). However, BP did not affect respiratory burst activity. Indeed, exposure to PMA triggered production of reactive oxygen intermediates in both untreated and BP-treated monocytes, and the levels of reactive oxygen intermediates found after PMA treatment did not statistically differ between BP-exposed and unexposed cells (Fig. 7). In addition, the cellular fluorescence values reflecting basal levels of reactive oxygen intermediates in the absence of PMA were similar in both BP-treated and untreated monocytes.

**PAHs inhibit M-CSF-triggered formation of adherent macrophagic cells from monocytes**

To determine whether the inhibitory effects of PAHs such as BP observed in GM-CSF-treated monocytes may also occur during GM-CSF-unrelated macrophagic differentiation, we have examined the action of 10 μM BP on M-CSF-mediated macrophagic differentiation. As shown in Fig. 8A and in agreement with

<table>
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<td>631.8 ± 229.4</td>
<td>577.8 ± 194.6</td>
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<td>CD11c (n = 9)</td>
<td>284.3 ± 121.4</td>
<td>148.2 ± 81.5b</td>
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<tr>
<td>CD14 (n = 11)</td>
<td>53.9 ± 73.6</td>
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<tr>
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<td>CD36 (n = 6)</td>
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<td>17.4 ± 7.1</td>
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<td>20.1 ± 17.2b</td>
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<td>HLA class II (n = 9)</td>
<td>126.2 ± 54.3</td>
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Table I. Phenotypic analysis of cultured monocyte cells either untreated or exposed to BP.

*a* Monocytes were cultured with GM-CSF for 6 days in the absence or presence of 10 μM BP. Phenotype analysis was then performed as described in Materials and Methods. Results are expressed as MFI and are the means ± SD of n independent experiments.

*bp < 0.05 when compared with BP-untreated cells.
previous reports (25), blood monocytes maintained for 6 days in the presence of M-CSF developed into adherent macrophagic cells displaying an elongated or stellate morphology. In the presence of $10^6$/H9262 M BP throughout the culture, the formation of adherent macrophagic cells was markedly diminished, and most of the cells remained as nonadherent cells in the culture medium (Fig. 8 A).

This loss of adherent macrophagic cells was fully illustrated using the WST-1 adhesion assay, because adherent monocytic cell counts in BP-treated cells were found to represent $20.8 \pm 15.1\%$ of the values detected in untreated counterparts ($n = 7, p < 0.05$, when compared with untreated cells). In addition, BP exposure was found to down-regulate surface levels of the macrophagic markers CD71, CD16, and CD64 in M-CSF-treated monocytic cells, whereas expression of CD14 remained unchanged (Fig. 8 B). It also reduced cellular endocytosis of FITC-dextran (data not shown).

**PAHs exert inhibitory effects toward differentiated macrophages**

To analyze PAH effects on differentiated macrophages, blood monocytes were first cultured with GM-CSF for 6 days and the macrophages obtained were then maintained in the absence or presence of $10 \mu$M BP for 4 days. Treatment by the PAH led to a loss of adherent macrophagic cells; indeed, the WST-1 adhesion assay indicated that adherent monocytic cell counts in BP-exposed macrophages corresponded to $48 \pm 19.7\%$ of the values found in untreated cells ($n = 10, p < 0.05$, when compared with untreated cells). In addition, levels of CD71 and CD64 expression were reduced by 2.7- and 3.8-fold, respectively, in BP-treated macrophagic cells when compared with untreated counterparts ($n = 7, p < 0.05$).

The AhR inhibitor $\alpha$-naphtoflavone counteracts PAH inhibitory effects toward macrophagic differentiation from monocytes

We finally investigated the putative involvement of AhR in PAH-mediated inhibitory effects toward macrophagic differentiation using the AhR antagonist $\alpha$-naphtoflavone (9, 33). We first verified that AhR was present and functional in our monocytic cell cultures. As shown in Fig. 9A, mRNAs of AhR and of its cofactor ARNT were detected in cultured monocytic cells as well as in human hepatocytes used here as positive cellular controls (6). Moreover, treatment of monocytic cells with $10 \mu$M BP was demonstrated to markedly up-regulate CYP1A1 expression (Fig. 9B), indicating that AhR was most likely fully functional in our monocytic cell culture system because this receptor is well-known to play a key role in PAH regulation of CYP1A1 (7).
6-day cultures revealed that \( \alpha \)-naphtoflavone was capable of markedly counteracting the inhibitory effects of BP toward the formation of adherent macrophagic cells (data not shown). In agreement with these morphological observations, data from WST-1 adhesion assays (Fig. 10A) indicated that adherent macrophagic cell numbers were similar whether the cells were untreated, exposed to \( \alpha \)-naphtoflavone alone, or cotreated with the combination of \( \alpha \)-naphtoflavone and BP. In contrast, exposure to BP markedly diminished the adherent monocytic cell fraction as already reported above. Moreover, addition of \( \alpha \)-naphtoflavone suppressed the down-regulation of surface levels of the macrophagic marker CD71 occurring in response to BP (Fig. 10B).

**Discussion**

Many PAHs are well recognized as potent immunosuppressive agents (9–12). Although lymphocytes have been shown to be targets (5), there is growing evidence that monocyte-derived cells such as myeloid dendritic cells and macrophages are also directly implicated in PAH immunotoxicity. Indeed, PAHs strongly impair functional differentiation and maturation of human monocytes into myeloid dendritic cells (24), and various experimental results indicate that functional capacities of macrophages are altered by PAHs (16–19). Our present data fully support the view that monocytic cells are important targets for PAHs, because we demonstrated that these chemicals inhibit GM-CSF- and M-CSF-mediated generation of functional macrophagic cells from human monocytes. Thus, we have found through microscopic examination of cultures and the use of an adhesion cell count assay that exposure to PAHs such as BP, MC, and DMBA during GM-CSF-triggered macrophagic differentiation decreased formation of adherent monocytic cells and prevented acquisition of characteristic morphological features of macrophages. BP was also demonstrated to exert a similar action in M-CSF-treated monocytic cultures. This loss of adhesion ability might be linked to altered expression of some integrins such as CD29, CD49e, and CD49f triggered by BP treatment in macrophagic cells. Interestingly, the effect of BP on cell adhesion was dose dependent and the active BP concentrations, i.e., 0.5–10 \( \mu \)M, were in the range of those known to affect PAH-sensitive cells such as oocytes, lymphocytes, and vascular smooth cells (4, 5, 34). BP effects persisted at least up to day 10 of culture, suggesting that BP continuously inhibited macrophagic differentiation from monocytes rather than simply delaying it. PAH exposure was associated with decreased expression of surface phenotypic markers usually up-regulated in macrophagic cells, especially CD71. Functional macrophagic properties such as endocytosis, phagocytosis, cytokine production, and APC activity...
were also impaired in PAH-exposed monocyctic cells. It is noteworthy that inhibitory effects of PAHs toward macrophagic generation were observed in both GM-CSF- and M-CSF-exposed monocyte cultures. This makes unlikely a specific interaction of PAHs with GM-CSF- or M-CSF-restricted transduction pathways and rather supports a general inhibitory effect of PAHs toward monocyctic differentiation pathways, whatever the cellular or molecular factor initially triggering this differentiation process. The fact that PAHs also inhibit formation of functional dendritic cells from blood monocytes upon the action of GM-CSF and IL-4 (24) fully supports this conclusion. In addition, it is noteworthy that BP was demonstrated to exert inhibitory effects toward macrophages previously differentiated upon the action of GM-CSF, thus resulting in decreased cell adhesion and down-regulation of the macrophagic markers CD71 and CD64. This suggests that PAHs can affect both the generation of macrophages and the properties of differentiated macrophages. These two effects may add up, leading to a major impairment of the monocytic/macrophagic cell lineage in response to PAHs.

Inhibition of macrophagic generation in PAH-exposed monocyte cultures was not associated with a nonspecific toxicity due to PAHs because we did not find any loss in cell viability in response to BP treatment. The observation that levels of surface phenotypic markers such as CD11b, CD36, CD40, CD14, and HLA class II molecules were not significantly altered in response to BP also argues against a major PAH toxicity. Moreover, this conclusion is supported by the preservation of PMA-triggered burst respiratory activity in BP-treated monocyctic cells, indicating that some of the monocyctic functions were not affected by PAHs. In addition, BP treatment did not elicit a major oxidative stress in cultured monocytes because similar cellular basal formation of reactive oxygen intermediates was found in both untreated and BP-treated monocyctic cells. A major oxidative toxic insult, which has already been shown to occur in response to PAHs in cell types such as vascular smooth muscle cells (34), was consequently not likely operating in our cell culture system. It is also noteworthy that BP exposure did not result in a marked apoptosis induction because apoptotic cell number in response to BP did not overrun 10% of total monocyctic cell population as assessed by Hoechst 33342 labeling of apoptotic nuclei. Similarly, BP and the halogenated arylhydrocarbon 2,3,7,8-tetrachlorodibenzop-dioxin failed to induce apoptosis in cultured monocyctic RAW 264.7 and U937 cells (35, 36), whereas PAHs are well-known to induce apoptosis in murine pre-B cells (15). Such data suggest that apoptotic effects of PAHs depend on cell types.

The cellular and molecular mechanisms underlying the inhibitory action of PAHs toward macrophagic differentiation remain to be clarified. The AhR, already known to mediate many immunosuppressive effects of PAHs (5, 15), is most likely involved because 1) this receptor and its cofactor ARNT are present in cultured monocytes in agreement with a previous report (37) and are fully functional as assessed by RT-PCR analyses of CYP1A1 expression, 2) among PAHs such as BP, DMBA, MC, and BeP, only the latter, which does not interact with AhR in contrast with the others (38), failed to down-regulate the formation of adherent macrophagic cells, and 3) the AhR antagonist α-naphtoflavone counteracted BP action on the macrophagic differentiation pathway. Therefore, through interacting with AhR in monocytic cells, PAHs might activate unidentified factors that hamper macrophage formation or, alternatively, might down-regulate factors required for macrophagic differentiation. Beyond PAH effects, the putative physiological role of AhR in monocytic cells may be worth considering because one might hypothesize that endogenous AhR ligands such as bilirubin (39) would negatively regulate macrophagic differentiation. More generally, our data fully support the idea that AhR may play a role in differentiation processes as recently suggested (40, 41). Further studies are certainly required to investigate this point.

Owing to the major role played by macrophages in innate and acquired immune defense, inhibition of their differentiation from monocytes in response to PAHs might significantly contribute to the potent immunosuppressive properties of these environmental contaminants. This conclusion agrees with numerous data obtained from PAH-treated mice, pointing out an implication of macrophagic cells in PAH immunotoxicity (16–19, 21). It is also supported by the phenotypic pattern of BP-treated monocytic cells, especially the down-regulation of costimulation molecules such as CD80 and CD86 involved in Ag presentation to lymphocytes, the decreased expression of Fcγ receptors such as CD16 and CD64 acting in Ab-dependent phagocytosis and cytotoxicity, and the diminished levels of integrins such as CD11a, CD11c, CD29, CD49e, and CD49f responsible for cell-cell interactions, cell adhesion, and migration. Moreover, BP-related alteration of endocytosis, phagocytosis, and allogeneic T cell stimulation likely impairs other macrophagic functions such as Ag processing and presentation and apoptotic cell clearance, whereas abolition of TNF-α production may compromise the development of local or
systemic inflammation. Interestingly, monocytes, whose differentiation pathways toward dendritic cells (24) or macrophages appear as key targets for PAHs, are also included in the main cell types activating PAHs into carcinogenic metabolites (42). Taken together, these data highlight the probable crucial role of monocytes in global pathogenesis of PAH toxicity. In addition, it is noteworthy that the lowest concentrations of BP active in vitro on monocytes, i.e., 0.5–1 μM (125–250 ng/g), are close to those found in charcoal-broiled foods (up to 50 ng/g) (43) or in the mainstream smoke of a cigarette (20 ng). The idea that humans may be exposed to PAH concentrations affecting monocytic cells.

In summary, our data indicate that exposure to PAHs strongly impairs differentiation of human monocytes into macrophages. This inhibitory effect, which likely involves, at least partly, activation of the AhR, may contribute to the potent immunotoxicity of PAHs.

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References