Dendritic cells (DCs) are critically important for the development of immune responses to various stimuli. Several major subsets of DCs are currently recognized: conventional (cDCs) and plasmacytoid DC (pDCs). These subsets although share some common progenitors, differ in many biological characteristics. In mice, pDCs are distinguished from cDCs by their expression of B220, Siglec-H and lack of MHC class II. pDCs are primarily found in lymphoid organs and have a unique role in the regulation of immune responses to various stimuli. Several major subsets of DCs—pDCs.

Aryl hydrocarbon receptor (AhR) has an important role in the regulation of cell responses to different environmental stimuli, as well as to various endogenous ligands. Although AhR was previously implicated in the regulation of dendritic cell (DC) activation, very little is known about its potential role in the development of these cells. Here we report our unexpected findings that AhR may regulate the differentiation of plasmacytoid DCs (pDCs). Agonist of AhR markedly decreased the generation of pDCs in vitro, whereas the AhR antagonist had an opposite effect. The differentiation of conventional DCs (cDCs) was not affected. AhR-knockout mice had a substantial accumulation of pDCs in peripheral lymphoid organs; whereas no changes in cDCs were seen. Thus, this study has identified AhR as a transcription factor involved in the development of one population of DCs—pDCs.

**RESULTS AND DISCUSSION**

**Lack of AhR promotes pDC development in vivo**

To investigate the possible role of AhR in DC differentiation, we first used AhR-KO mice. pDCs were defined as CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup>Siglec-H<sup>+</sup>, CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup>Siglec-H<sup>+</sup>, or CD11c<sup>+</sup>CD11b<sup>+</sup>Bst2<sup>+</sup>Ly6c<sup>+</sup> cells (Figure 1a and data not shown). cDCs were defined as CD11c<sup>+</sup>CD11b<sup>+</sup>CD11b<sup>+</sup>Ly6c<sup>+</sup>I-Ab<sup>+</sup> cells. Gating strategy is shown in Supplementary Figure S1. The absence of AhR did not significantly affect the total number of lymph node (LN) cells or splenocytes (Figure 1b). The proportion and absolute number of pDCs in spleens of AhR-KO mice was higher than in wild-type (WT) mice. In contrast, no differences were found in the presence of cDCs and Gr-1<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages (Figure 1c). Similar results were obtained during the analysis of DC populations in LNs (Figure 1d). AhR could affect HPC or regulate pDC development via intrinsic mechanisms or by affecting bone marrow (BM) microenvironment. To address this question, enriched HPCs, obtained from the BM of WT and AhR-KO mice, were cultured for 9 days with Flt3-L, which supports the differentiation of both cDCs and pDCs. We observed a significantly higher proportion of pDC, generated from ahr<sup>−/−</sup> mice than from WT mice. No differences in the presence of cDC were found (Figure 1e).

To test their functional activity, pDCs, generated from AhR-deficient HPCs, were isolated on day 9 and stimulated with CpG-A ODN. pDCs, generated from WT and AhR-deficient HPCs, had an...
equally high level of IFN-γ production (Figure 1f) thus indicating that the population of cells, with the phenotype of pDCs accumulated in AhR-deficient mice, demonstrated the functional characteristic of pDCs. These data suggested that AhR could negatively regulate the differentiation of the pDC subset of DCs without affecting cDCs.

**Regulation of AhR in vitro affects pDC differentiation**

The expression of ahr was gradually increased during in vitro differentiation of DCs from bone marrow HPC in the presence of Flt3-L. The upregulation of AhR repressor ahr was much smaller, and, as a result, the ahr/ahrr ratio increased substantially by day 9 (Figure 2a). To investigate the role of AhR signaling in differentiation of pDCs further, we activated AhR with its specific ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) during 9-day HPC culture with Flt3-L. TCDD did not affect the total number of cells generated from HPC (Figure 2b), but it caused a substantial decrease in the proportion of pDCs (Figures 2c and d, top panel). In contrast, the presence of cDCs was not significantly changed (Figure 2d, bottom panel). We used AhR antagonist CH223191. A significant increase of pDCs was observed, whereas no effect on cDCs was found (Figure 2e). Side-by-side experiments with TCDD and CH223191 using the same cell markers confirmed these observations (Supplementary Figure S2). TCDD and CH223191 at selected concentration did not induce apoptosis of pDCs (Figure 2f) or cDCs (data not shown) generated in vitro. To clarify the effect of AhR agonist on cDC development, we differentiated DCs from HPCs in the presence of GM-CSF. Under these conditions, only cDCs are generated. TCDD had no effect on cDC differentiation (Figure 2g). Thus, it appears that the activation of AhR had an inhibitory effect on pDC differentiation in vitro, but did not affect cDCs.

Thus, this is a first report demonstrating a possible role of AhR in the regulation of pDC differentiation. Although AhR was previously implicated in the regulation of DC activation, very little is known about its potential role in the differentiation of these cells. Previous study suggested a redistribution of DCs after the treatment of mice with TCDD. Our data demonstrated that AhR may selectively inhibit differentiation of pDCs but have no effect on cDC differentiation. The mechanism of this effect is not clear and needs further investigation.

**METHODS**

**Mice**

All mouse experiments were approved by the University of South Florida Institutional Animal Care and Use Committee. Female C57BL/6 mice (age 6–8 weeks) were obtained from the National Cancer Institute and C57BL/6-Ahrtm1.1(AHR)Arte and pDC mice from Taconic (Hudson, NY, USA). All mice were housed in pathogen-free units of the vivarium in University of South Florida.

**Reagents**

A F4/80 (CLA3-1) antibody was purchased from Serotec (Raleigh, NC, USA); CD11c (N418), Bst2 (129c) and Siglec-H (eBio440c) from Ebioscience (San Diego, CA, USA); and antiphycoerythrin (anti-PE) microbeads from Miltenyi.
**Figure 2** AhR negatively regulates pDC development in vitro. (a) The relative mRNA levels of ahr, ahrr and arnt in HPC culture with Flt3-L on days 3, 6 and 9. The ratio of ahr to ahrr was present. Data are presented as mean ± s.e.m. of three independent experiments. (b) HPCs were cultured with 100 ng ml⁻¹ Flt3-L for 9 days in the presence of 10 nm TCDD or vehicle. (b) The total number of cells generated from HPC. (c) Typical FACS plots of cells gated on CD11b⁻ cells from three independent experiments. The percentage of pDCs from all live cells is shown. Right panel—proportion of cells. Data are presented as mean ± s.e.m. (n = 3 replicates). (e) HPCs were cultured with Flt3-L in the presence of 1 μM CH223191 or vehicle for 9 days. Left panel—representative FACS plots of cells gated on CD11b⁻ cells from three independent experiments. The percentage of pDCs from all live cells is shown. Right panel—proportion of cells. Data are presented as mean ± s.e.m. (f) Apoptosis was measured using staining with DAPI and Annexin-V within the population of pDCs generated from BM progenitors in the presence of TCDD and CH223191 as described in Figures 2d,e. Mean and s.e.m. are shown (n = 3). (g) HPCs cultured with 20 ng ml⁻¹ GM-CSF for 5 days in the presence of 10 nm TCDD, 1 μM CH223191 or vehicle. Data are presented as mean ± s.e.m. from three independent experiments.

Biotec (Auburn, CA, USA). All other antibodies were purchased from BD (Franklin Lakes, NJ, USA). The recombinant GM-CSF and IL-4 are from R&D Systems (Minneapolis, MN, USA). CpG ODN type A (1585) and CpG ODN type B (1668) from Invitrogen (San Diego, CA, USA). TCDD and CH 223191 were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA) and TOCRIS (Minneapolis, MN, USA).

**Generation of DCs and ELISA**

HPCs were enriched with a lineage depletion kit (Miltenyi) and cultured with 100 ng ml⁻¹ Flt3-L for 9 days or with 20 ng ml⁻¹ GM-CSF for 5 days. pDCs were purified with a siglec-H-PE antibody, followed by anti-PE microbeads (Miltenyi) (purity > 95%). In all, 50,000 cells were stimulated with 20 ng ml⁻¹ CpG-A ODN 16 h. IFN-γ was measured in supernatants using the mouse IFN-γ ELISA kit (Ebioscience).

**Real-time PCR**

mRNA was extracted with a Qiagen RNeasy kit (Valencia, CA, USA) and reverse-transcribed into cDNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Grand Island, NY, USA). qRT-PCR was performed with SYBRgreen using the following primers: ahr (forward: 5′-AGG GCC AAG AGC TTC TTT TCT TTT TCT CATT T-3′; reverse: 5′-CTG TCG TCG TGG GCC TCT CATT T-3′); arnt (forward: 5′-AAG CAG ACA AAG TAA CCA CTA TCT TAC G-3′; reverse: 5′-ATC AAA TGT TTC AGT TCC TGA TCA GT-3′); ahrr (forward: 5′-TCCCG GTCGG GAGGA-3′; reverse: 5′-TGC TGA TCG TCA AAA GAT TAC AAT AAG GT-3′).
CAT C-3) hprt served as an interior control (forward: 5'-TTC CTC GATG TGA TGA AGG A-3'; reverse: 5'-CCA GCA GGT CAG CAA AGA ATT-3').

Statistical methods
The data were analyzed with a two-tailed Student t-test. P-values <0.05 were considered to be statistically significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Author contributions: HL performed experiments, analyzed the results and wrote the paper; IR performed experiments; DIG designed the experiments, analyzed the results and wrote the paper.

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