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*J Immunol* 2009; 183:66-74; Prepublished online 17 June 2009;
doi: 10.4049/jimmunol.0802997
http://www.jimmunol.org/content/183/1/66

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Aryl Hydrocarbon Receptor Activation Inhibits In Vitro Differentiation of Human Monocytes and Langerhans Dendritic Cells

Barbara Platzer,‡ Susanne Richter,‡ Doris Kneidinger, † Darina Waltenberger, † Maximilian Woisetschläger,‡ and Herbert Strobl³

The transcription factor aryl hydrocarbon receptor (AhR) represents a promising therapeutic target in allergy and autoimmunity. AhR signaling induced by the newly described ligand VAF347 inhibits allergic lung inflammation as well as suppresses pancreatic islet allograft rejection. These effects are likely mediated via alterations in dendritic cell (DC) function. Moreover, VAF347 induces tolerogenic DCs. Langerhans cells (LCs) are immediate targets of exogenous AhR ligands at epithelial surfaces; how they respond to AhR ligands remained undefined. We studied AhR expression and function in human LCs and myelopoietic cell subsets using a lineage differentiation and gene transduction model of human CD34+ hematopoietic progenitors. We found that AhR is highly regulated during myeloid subset differentiation. LCs expressed highest AhR levels followed by monocytes. Conversely, neutrophil granulocytes lacked AhR expression. AhR ligands including VAF347 arrested the differentiation of monocytes and LCs at an early precursor cell stage, whereas progenitor cell expansion or granulopoiesis remained unimpaired. AhR expression was correlated with the transcription factor PU.1 during myeloid subset differentiation. VAF347 inhibited PU.1 induction during initial monocytic differentiation, and ectopic PU.1 restored monocyte and LC differentiation in the presence of this compound. AhR ligands failed to interfere with cytokine receptor signaling during LC differentiation and failed to impair LC activation/maturation. VAF347-mediated antiproliferative effect on precursors undergoing LC lineage differentiation occurred in a clinically applicable serum-free culture model and was not accompanied by apoptosis induction. In conclusion, AhR agonist signaling interferes with transcriptional processes leading to monocyte/DC lineage commitment of human myeloid progenitor cells. The Journal of Immunology, 2009, 183: 66–74.

Recently, it was shown that depending on the nature of the applied ligand, AhR signaling can either suppress autoimmunity via induction of regulatory T cells (Treg) (i.e., TCDD) (9), or lead to worsened autoimmunity by interfering with Treg and boosting IL-17-producing helper T (T_{h17}) cell development (i.e., 6-formylindolo[3,2-b]carbazole) (10). The low m.w. compound VAF347 represents a recently identified AhR ligand with potent anti-inflammatory activity (11). VAF347 exceeded suplatastat tosylate, a commonly used anti-asthma drug, in suppressing lung inflammation in a murine model of allergic asthma, an effect mediated at least in part via altered dendritic cell (DC) function (12). Similarly, oral administration of VAG539, a compound that is converted to VAF347 in vivo, promotes long-term graft acceptance and active tolerance in BALB/c mice transplanted with MHC-mismatched pancreatic islet allografts (13). DCs seemed to play a key role in this model, since transfer of DCs but not T or B lymphocyte subsets from VAG539-treated hosts into newly transplanted mice resulted in donor specific graft acceptance. Furthermore, ex vivo VAF347 treatment of bone marrow-derived DCs resulted in tolerogenic DCs that suppressed islet graft rejection and they reduced OVA-specific T cell responses in OVA-immunized mice (13). In line with this, VAF347 inhibited IL-6 production and Ag-specific T cell priming by human monocyte-derived DCs (moDC) (12). Monocytes express AhR and moDC differentiation can be inhibited by certain AhR ligands (14, 15). However, because TCDD failed to impair moDC differentiation, non-AhR-mediated effects were likely involved (15).

Langerhans cells (LCs) form dense networks in epithelia and are therefore immediate targets of environmental substances and toxins including exogenous AhR ligands. LCs are exposed to specific microenvironmental signals in epithelia, such as TGF-β1, a key
cytokine required for their differentiation from monocyctic cells (16–18). They traffic from the epithelium to lymph nodes even under noninflamed conditions, and LCs were previously implicated in Treg induction (19). Whether these epithelial DCs express AhR and how they functionally respond to AhR ligands is unknown.

Given its potential clinical application, we rationalized that a detailed study on AhR signaling in human LCs/DCs is of substantial relevance. In this study, we demonstrated that AhR agonists such as VAF347 impair LC/DC function by inhibiting their differentiation from monocyctic precursors, rather than by interfering with DC maturation. Furthermore, we identified PU.1 as a key molecular target by which AhR agonists control myeloid/DC sublineage differentiation.

Materials and Methods

Cytokines and reagents for in vitro cultures of CD34+ cells

Human stem cell factor (SCF), thrombopoietin, G-CSF, and M-CSF, TNF-α, and IL-4 were purchased from PeproTech; fms-related tyrosine kinase 3 ligand (Flt3L) was obtained from Amgen; TGF-β1 was purchased from R&D Systems; IL-6 and GM-CSF were provided by Novartis Institutes of BioMedical Research. The AhR-ligands β-naphthoflavone (β-NF) and TCDD were purchased from Crescent Chemical Company. VAF347 (17) was provided by Novartis Institutes for BioMedical Research. IL-1β and IFN-γ were purchased from PeproTech. CD40L was provided by Amgen.

Cell isolation and cultures of CD34+ cord blood cells

Cord-blood samples from healthy donors were collected during healthy full-term deliveries. Approval was obtained from the Medical University of Vienna institutional review board for these studies. Informed consent was provided in accordance with the Declaration of Helsinki. CD34+ cells, CD14+ monocytes, and neutrophils were isolated as described (20). CD11a+ LCs were isolated from epidermal cell suspensions from healthy individuals undergoing reconstructive surgery as described (21). LC purity exceeded 95%. In vitro generation of LCs, monocytes, and granulocytes as well as progenitor expansion was performed as described (18, 22). In brief, CD34+ cord blood cells were cultured for 2 to 3 days under progenitor expansion conditions before subculturing with lineage-specific cytokines. Cytokine supplementation: progenitor cell expansion cultures, Flt3L, SCF and thrombopoietin, each at 50 ng/ml; monocyte cultures, M-CSF (100 ng/ml), IL-6 (20 ng/ml), Flt3L (50 ng/ml) and SCF (20 ng/ml); LC cultures, GM-CSF (100 ng/ml), SCF (20 ng/ml), Flt3L (50 ng/ml), TNF-α (2.5 ng/ml), and TGF-β1 (0.5 ng/ml); granulocyte cultures, G-CSF (100 ng/ml) and SCF (20 ng/ml). For MoDC generation, CD14+ monocytes were isolated from human peripheral blood using CD14-PE (Caltag Laboratories) and MACS anti-PE magnetic beads (Miltenyi Biotec). Cells were subsequently cultured in RPMI 1640 (plus 10% FCS) supplemented with GM-CSF (200 ng/ml) and IL-4 (25 ng/ml) for 7 days. The AhR ligands VAF347, TCDD, and β-NF were dissolved in DMSO and 10 mM stock solutions were further diluted in medium. Medium, cytokines, and AhR ligands were renewed every 4 days. Cells were counted using CASY counter, Model TT (Schräfe Systems, Innovatis).

Flow cytometry

Flow cytometry staining and analysis was performed as previously described (23). For detecting apoptotic cells AnnexinV-FITC (Caltag Laboratories) was used as recommended. For detecting dead cells 7-aminoactinomycin D (Sigma-Aldrich) was used. Marine mAbs of the following specificities were used: FITC-conjugated mAbs specific for CD1a and CD15 (BD Biosciences); PE-conjugated mAbs specific for Langerin (Immunotech), CD11b (BD Biosciences), myeloperoxidase, (Caltag Laboratories), Lactoferrin (Caltag Laboratories), HLA-DR (BD Pharmingen); biotinylated mAbs specific for CD86 and CD11b (BD Pharmingen); second step reagent was streptavidin-PerCP (BD Pharmingen); allophycocyanin-conjugated mAbs specific for CD1a, CD83 (BD Pharmingen), and CD14 (Caltag Laboratories). The flow cytometric acquisition and data analysis was performed using a FACSCalibur and CellQuest Pro software (BD Biosciences).

Real-time PCR

Pre-expanded CD34+ cells (3 days) were cultured under monocyte-inducing conditions and K562 cells were cultured in RPMI 1640 plus 10% FCS in the presence or absence of VAF347. Equal amounts of cells (5 × 10^7 to 1 × 10^8) were harvested and total RNA was isolated using RNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions including DNase I treatment to remove DNA contaminations. Purified RNA was subjected to CDNA synthesis using oligo-dT-primers (MWG) and reverse transcriptase (M-MLV RT H+; Fermentas) according to the manufacturer’s instructions. For real-time RT-PCR analysis of human ex vivo isolated LC, RNA was isolated and cDNA was prepared using Random-Hexamers. Analysis was performed using LightCycler instrument (Roche Diagnostics), and Platinum SYBR Green PCR SuperMix-UDG (Invitrogen). A melting curve analysis was performed to verify the specificity of the amplified PCR products. Relative PU.1 gene transcription, 293T cells were transiently transfected with the following vectors: (I) pPU.3-TK-luc or pTK-luc, (II) pRL-TK, and (III) pBMN-IRES-GFP (CTRL) or pBMN-PU.1-IRES-GFP. Vectors pTK-luc and pRL-TK were purchased from BD Bioscience. pPU.3-TK-luc containing a <3 PU.1 consensus binding site, provided by T. Graf (Centre for Genomic Regulation, Barcelona, Spain), was described previously (26). VAF347 was added as indicated 36 h post transfection, and cells were harvested after additional 12 h. Luciferase activity was measured using Dual Luciferase Reporter Kit from Promega on a Lumat LB 9501 (Berthold). Relative luciferase activity was calculated as the ratio of firefly luciferase activity induced by pPU.3-promoter normalized to Renilla luciferase activity induced by the constitutively active pRL-TK vector.

Retroviral constructs and gene transduction

The retroviral construct pBMN-PU.1-IRES-GFP as well as the gene transduction procedure of CD34+ progenitor cells was previously described (24). K562 NF-κB reporter cells were generated using an identical procedure as previously described for U937 cells (27). Lentiviral GPZP-short-hairpin (sh)RNA-GFP directed against the AhR and a control nonsilencing sequence was purchased from Open Biosystems, and subcloned into the retroviral MSCV-LMP-GFP vector backbone (Open Biosystems) using Xhol and EcoRI site.

Statistical analysis

Statistical analysis was performed using the paired, 2-tailed Student t test; p values <0.05 were considered significant.

Results

AhR is differentially expressed by human myelopoietic cell subsets

Because it was not known whether or how AhR expression is regulated during myelopoietic cell differentiation, we first analyzed AhR expression in different myeloid cell types. We generated granulocytes, monocytes, and LC-type dendritic cells (LCs) from CD34+ cells as previously described (20, 22) (Fig. 1A) and analyzed AhR protein expression by Western blotting (Fig. 1B). AhR protein was found to be expressed at high levels in LCs and at intermediate levels in monocytes, while granulocytes displayed only low to undetectable levels of AhR (Fig. 1B). In comparison, expanded progenitor cells showed low to intermediate levels of AhR (Fig. 1B). In line with the observed inverse expression of AhR by in vitro generated monocytes vs granulocytes, substantial
Selective impairment of LC and monocyte differentiation by AhR ligands

We generated LCs, monocytes, or granulocytes from progenitors as above (Fig. 1A) in presence or absence of VAF347. Addition of VAF347 strongly inhibited the generation of CD1a<sup>+</sup>Langerhin<sup>+</sup> LCs (Fig. 2A) as well as impaired the generation of CD14<sup>+</sup> CD11b<sup>+</sup> monocytes (Fig. 2C). Fully differentiated CD11b<sup>+</sup> CD14<sup>+</sup> monocytes were selectively lacking in the presence of VAF347, while percentages of cells showing an earlier monocytic phenotype (CD14<sup>+</sup>CD11b<sup>−</sup>Lin<sup>−</sup>) increased in VAF347-treated cultures relative to control. Conversely, progenitor cell differentiation to CD15<sup>+</sup>Lactoferrin<sup>+</sup> granulocytes remained unimpaired (Fig. 2D, FACS and bar diagrams). Activation of the AhR signaling pathway can be induced by a variety of compounds. All analyzed AhR ligands (i.e., β-naphthoflavone, TCDD, and VAF347) were similarly effective in impairing LC and monocyte generation (Fig. 2A and data not shown). Cyp1a1 up-regulation, which is widely used as indicator of AhR activation, could be detected in TCDD or VAF347 treated cells (Fig. 2B). Therefore, AhR agonists exerted selective inhibitory effects on monocytes and LCs.

To support that AhR activation is involved in the inhibition of myeloid progenitor cell differentiation, we performed retroviral shRNA-mediated AhR knock-down experiments. The retroviral shRNA silencing vector directed against AhR (shAhR) encoded GFP, thus allowing us to identify transduced cells using flow cytometry. After gene transduction, progenitor cells were cultured for 7 days in medium supplemented with M-CSF, FL, IL-6, and SCF to generate CD14<sup>+</sup>CD11b<sup>+</sup> monocytes. Afterward, gated GFP<sup>+</sup> cells were analyzed for CD14<sup>−</sup>CD11b<sup>−</sup> expression. In control cultures VAF347 reduced % CD14<sup>+</sup>CD11b<sup>+</sup> cells (94 vs 52% of generated cells), whereas this inhibitory effect of VAF347 was diminished in cells transduced with shAhR (89 vs 82%). Control experiments confirmed reduced AhR protein expression in shAhR transduced cells (Fig. 2E, Western blot). These observations support that AhR is functionally required for VAF347-mediated inhibition of myelopoietic progenitor cell differentiation.

VAF347 impairs the proliferation of cells undergoing monocyte or LC differentiation

CD34<sup>+</sup> cells strongly proliferate during their differentiation into monocytes, LCs, and granulocytes or during progenitor cell expansion. Cell yields were lower in monocyte and LC cultures treated with VAF347 compared with untreated cultures (Fig. 3A, Mo, LC). Calculation of absolute cell numbers of CD14<sup>+</sup> cells revealed a substantial inhibition of CD14<sup>+</sup> cell generation by VAF347 (Fig. 3A, LC, right bar diagram). Conversely, cell numbers in granulocyte or progenitor cell expansion cultures were not significantly influenced by the AhR agonist (Fig. 3A, Gr and Exp., prog.). Suppression of cell yields by AhR was not due to enhanced apoptosis induction (Fig. 3B). Next, CD34<sup>+</sup> cells were labeled with the membrane dye PKH26, and were subsequently cultured under LC, monocyte, granulocyte, and progenitor expansion conditions with or without the compound. PKH26 dye dilution, which corresponds to cell proliferation, was assessed by flow cytometry after 5 to 7 days. VAF347 reduced cell proliferation in monocyte- and LC-cultures, whereas cell proliferation in granulocyte- or progenitor expansion- cultures remained unimpaired (Fig. 3C). Separate gating on CD1a<sup>+</sup> and CD1a<sup>−</sup> cells present in LC cultures revealed that the proliferation of cells giving rise to CD1a<sup>+</sup> cells was specifically inhibited by VAF347 (Fig. 3C). Conversely, CD1a<sup>−</sup> cells were not inhibited by VAF347, because CD1a<sup>−</sup> cells from VAF347-treated LC cultures showed a similar PKH26 staining intensity as CD1a<sup>+</sup> or CD1a<sup>−</sup> cells from nontreated cultures.

**FIGURE 1.** AhR protein expression in different myeloid cell types. A. Schematic overview of in vitro generation of LCs, monocytes, granulocytes, and progenitor expansion from CD34<sup>+</sup> precursor cells. B. Comparison of AhR expression in different myeloid cell types using Western blotting. CD34<sup>+</sup> cells were cultured using the cytokine conditions depicted in A to generate the indicated cell types. C. AhR expression in human peripheral blood monocytes and granulocytes. Left blot, Equal cell numbers were used. Right blot, Actin levels were adjusted. D. AhR expression by ex vivo isolated LCs. Left, Western blot of LCs in comparison to CD34<sup>+</sup> cells isolated from human cord blood. Right, Real-time RT-PCR analysis of LCs vs peripheral blood monocytes. Bars represent relative levels of AhR mRNA. Results of two independent LC donor preparations (>95% cell purity) are shown.
FIGURE 2. Effects of AhR ligands on myeloid progenitor cell differentiation. A. AhR ligands VAF347, β-naphtoflavone (β-NF) and TCDD cause similar inhibition of LC generation. CD34⁺ progenitor cells were induced to differentiate into LCs (see Fig. 1A) in the presence or absence of 50 nM VAF347, 500 nM β-NF, or 10 nM TCDD for 7 days. Cultures were analyzed by FACS for percentages of CD1a⁺ cells (bar diagram, mean ± SD of four independent experiments) or analyzed for CD1a vs Langerin expression (representative dot plots are shown). B, VAF347- or TCDD-mediated up-regulation of Cyp1a1 in LC cultures. Relative levels of Cyp1a1 mRNA were measured by real-time RT-PCR at the time points indicated. C, VAF347 impairs the development of CD14⁺CD11b⁺ monocytes from CD34⁺ cells. Representative FACS diagrams are shown. Bars represent mean values (±SD) of six independent experiments. D, Granulocytes were generated from CD34⁺ cells in absence or presence of 50 nM VAF347. Generated cells were then analyzed for the differentiation markers CD15 vs intracellular Lactoferrin (LF). FACS diagrams: Left, CD15 vs isotype control; Middle and right, CD15 vs intracellular Lactoferrin. Bars represent mean percentages (±SD) CD15⁺Lactoferrin⁺ cells; n = 3 experiments. E, VAF347-mediated inhibition of monocyte differentiation is dependent on AhR. CD34⁺ cells were infected with a retroviral shAhR silencing vector encoding GFP. Gated GFP⁺ cells are analyzed for CD11b vs CD14 (representative dot blots are shown; n = 2). Western blot, AhR protein expression levels in FACS sorted GFP⁺ cells (shAhR vs nonsilencing control vector).
AhR activation inhibits Langerhans cell differentiation

Because it has been shown that the ectopic expression of PU.1 induces the development of monocytes from common myeloid progenitors at the expense of granulocytes (28), we next analyzed a possible mechanistic relationship between AhR activation and PU.1. As also observed for AhR (Fig. 1B), highest expression levels of PU.1 were observed in LCs followed by monocytes (Fig. 4A). We hypothesized that AhR-dependent inhibition of monocyte/LC differentiation is mediated by PU.1 antagonism. Thus, we asked whether AhR activation can inhibit PU.1-mediated gene expression. To test this possibility, we performed a luciferase reporter assay (Fig. 4B) using 293T cells. VAF347 failed to impair PU.1-mediated reporter gene induction (Fig. 4B), demonstrating that AhR does not directly interfere with PU.1-dependent gene expression. Next, we asked whether VAF347 treatment might interfere with PU.1 up-regulation during the initial differentiation of CD34+ cells into monocyteic cells. Thus, CD34+ progenitor cells were cultured in the presence or absence of VAF347 under monocyte-specific differentiation conditions and PU.1 mRNA expression was determined using real-time RT-PCR. Nontreated cells started to up-regulate PU.1 mRNA expression after 10 to 24 h. Treatment with VAF347 significantly inhibited PU.1 induction at 24 h (Fig. 4C, bar diagram). Therefore, VAF347-induced AhR signaling impairs PU.1 induction by myeloid progenitors during initial monocyteic cell differentiation. To affirm whether AhR agonist-mediated inhibition of PU.1 expression could be responsible for the inhibition of LC and monocyteic differentiation, CD34+ progenitors were transduced with a retroviral vector encoding PU.1-IRES-GFP or empty control (CTRL). Cells were then stimulated to undergo LC or monocyteic differentiation, and the effect of VAF347 addition was analyzed. PU.1-transduced cells vs nontransduced cells were identified by the expression of GFP, GFP+ cells were gated separately and analyzed for LC (CD1a\textsuperscript{high}Langerin\textsuperscript{−}) or monocyteic induction (CD14\textsuperscript{+}CD11b\textsuperscript{−}). In line with above data (Fig. 2), VAF347 inhibited LC generation from both CTRL transduced cultures as well as nontransduced GFP− cells (Fig. 4D).
FIGURE 4. VAF347 impairs monocyte/LC differentiation by inhibition of PU.1. A, PU.1 expression in different myeloid cell types. B, AhR activation does not interfere with PU.1-dependent promoter activity. 293T cells were cotransfected with pPU.1-TK-luc (3xPU.1 consensus site upstream of luciferase) or control (pTK-luc; not shown) and PU.1-IRES-GFP (ecPU.1) or empty vector (CTRL). Renilla vector was used as an internal control. Cells were harvested 12 h post VAF347 addition. Bars represent relative luciferase activity (±SEM) of four independent experiments. C, Real-time RT-PCR of PU.1 induction in monocytic precursors. Left diagram: Time kinetics analysis of one representative experiment; Right diagram, Bars represent mean values and SEM of four independent experiments (*, p = 0.0041; **, p = 0.005). D and E, CD34+ cells were infected with a retroviral vector encoding PU.1-IRES-GFP or empty vector pBMN-IRES-GFP (CTRL). Cells were then induced to differentiate into LCs or monocytes in the presence or absence of VAF347 (50 nm) and analyzed by flow cytometry. D, GFP+ cells and GFP− cells from day 7 LC cultures were gated on a separate diagram (data not shown), and both fractions were analyzed for the expression of CD1a and Langerin (upper and lower panels of FACS diagrams, one representative experiment is shown). E and F, Analysis of gene transduced cells from LC (E) and monocyte (F) cultures. Bars represent mean values (±SEM) of percentages CD1a+Langerin+ (E) or CD11b+CD14+ cells (F) among GFP+ (PU.1- or CTRL-transduced) cells (n = 4 independent experiments; *, p < 0.05; n.s. not significant). Strikingly, gating on PU.1 transduced cells (GFP+) revealed that addition of VAF347 fails to repress LC development from these cells. Ectopic PU.1 expression not only rescued LC differentiation in the presence of the compound but rather led to increased LC generation compared with PU.1 transduced cultures in the absence of the AhR agonist (Figs. 4D, upper panel and E). Similarly, ectopic PU.1 expression rescued VAF347-induced inhibition of monocyte development (Fig. 4F). Together these data demonstrated that ectopic PU.1 can overcome VAF347-mediated impairment of LC and monocyte differentiation.

VAF347-mediated impairment of LC generation cannot be explained by inhibition of TGF-β1 or of TNF-α/NF-κB signaling

We previously showed that TGF-β1 costimulation induces a PU.1high LC population, and that elimination of TGF-β1 from the culture system shifts the differentiation pattern of CD34+ progenitors from LCs to CD14+ monocytic precursor cells (24). Cross-talk between AhR and TGF-β1 signaling was previously described (29–32). Furthermore, AhR activation was shown to mimic TGF-β1 signaling in Treg induction (9). In line with above data (Fig. 2A), addition of VAF347 at culture onset strongly reduces percentages of LCs in a dose-dependent manner (Fig. 5A, empty bars), and reciprocal to this, CD11bdim/CD14dim monocytic precursor cells appeared (Fig. 5A, black bars and FACS diagrams). These latter cells showed lower CD11b and CD14 mean fluorescence intensity as compared with CD11b+ CD14+ monocytes generated in M-CSF/IL-6-supplemented cultures in the absence of VAF347 (compare Figs. 2C and 5A). Furthermore, they phenotypically resembled previously described CD11bhigh/CD14dim cells generated monocytic precursor cells in LC cultures in the absence of TGF-β1. These cells are generated in the absence of M-CSF and represent monocytic precursors (24). Because ectopic PU.1 strictly requires TGF-β1 for LC induction (24) and PU.1 transduction of progenitors was sufficient to overcome the VAF347-mediated impairment of LC differentiation (Fig. 4E), it was highly unlikely that the compound directly interferes with TGF-β1 signaling. In line with this, VAF347 failed to inhibit the induction of the well-established TGF-β1-inducible gene plasminogen activator inhibitor (PAI-1) (33) in K562 hematopoietic cells (Fig. 5B). Another signaling pathway important for DC differentiation from CD34+ cells is TNF-α-induced NF-κB activation. Because AhR was previously found to bind and block NF-κB p65 (34, 35), we tested whether VAF347 inhibits TNF-α-induced NF-κB activation in hematopoietic cells. Therefore, K562 cells were stably transduced with a 5× NF-κB-GFP reporter cassette. These cells were then stimulated with TNF-α in the presence or absence of VAF347 (Fig. 5C, left). TNF-α addition strongly induces GFP indicative of NF-κB activation, and VAF347 failed to impair GFP induction (Fig. 5C, left). Control experiments confirmed that VAF347 rapidly induces Cyp1a1 expression in K562 cells (Fig. 5C, right). These data support that AhR activation in response to VAF347 stimulation fails to inhibit TNF-α-induced NF-κB signaling in hematopoietic cells.

Impairment of differentiation but not maturation of LCs by AhR activation

Next, we studied whether AhR activation interferes with LC maturation. In vitro generated LCs were stimulated for 48 h with CD40L plus IL-1β plus IFN-γ (Fig. 5D) or LPS (data not shown) to induce up-regulation of costimulatory molecules indicative of LC maturation. Addition of VAF347 to differentiated LCs failed to impair the up-regulation of mature DC features such as MHC class II (HLA-DR), CD86, and CD83 (Fig. 5D). When VAF347 was added at culture onset and then maturation was induced after 7
days, decreased expression of these maturation markers was observed (data not shown). This impaired maturation was expected due to inhibition of CD1a/LC generation under this culture condition as demonstrated above (Figs. 2A and 5A). Thus, AhR activation severely impaired LC generation from precursors, but did not affect final LC maturation.

AhR ligation fails to inhibit monocyte to DC differentiation

Above data show that AhR activation inhibits LC differentiation from hematopoietic CD34⁺ progenitor cells (Fig. 2A). We further analyzed whether the differentiation of CD1a⁺ cells from CD14⁺ peripheral blood monocytes is inhibited by AhR activation. Thus, we cultured purified CD14⁺ peripheral blood monocytes in the presence of GM-CSF plus IL-4 and analyzed the effects of VAF347 on the generation of CD1a⁺CD14⁺ moDCs. As can be seen from Fig. 5E (FACS and bar diagrams), day 7 cultures in presence or absence of VAF347 contained similar high percentages of CD1a⁺CD14⁺ cells. Therefore, despite its strong inhibitory effect on CD1a⁺ LC generation (Fig. 2A), VAF347-mediated AhR activation fails to interfere with the generation of CD1a⁺ monocyte-derived DCs.

Discussion

The newly identified AhR agonist VAF347 was described as a potent immunosuppressive compound in a murine model of T cell-dependent allergic asthma (11, 12). This effect was likely mediated via impaired DC function, rather than by direct impairment of T
cells (12). Similarly, VAF347 administration can suppress murine allograft rejection, and induce tolerogenic DCs in vivo and in vitro (13). This raised the question how AhR agonist signaling might modulate DC function. Furthermore, studies on human DCS were lacking. Because murine and human leukocytes are known to be differentially susceptible to AhR-mediated effects (36), a detailed analysis of how AhR activation might interfere with human DC development and maturation is of particular relevance.

We demonstrated in this study that AhR activation by exogenous ligands selectively impairs monocyte and LC differentiation of myeloid progenitor cells, while granulopoiesis or progenitor cell expansion remained unimpaired. VAF347 exerted a specific anti-proliferative effect on precursors undergoing monocyte or LC lineage differentiation and this effect was not accompanied by apoptosis induction. We identified the transcription factor PU.1 as a key determinant for this effect. AhR activation inhibited PU.1 induction in monopoietic cells and arrested monocyte differentiation at a precursor cell stage (CD11bdim/CD14dim). Similarly, LC differentiation, known to derive in vitro from early monocytic cells (37, 38), was profoundly Impaired and arrested at a monocytic precursor cell stage (CD11bdim/CD14dim). Retroviral vector-mediated ectopic expression of PU.1 in progenitor cells was sufficient to fully restore monocyte or LC differentiation in the presence of an AhR ligand. We further demonstrated in this study that in vitro-generated granulocytes and peripheral blood neutrophils show undetectable AhR expression and, in line with this, AhR activation failed to interfere with granulopoiesis. Similarly, progenitor cell expansion and DC maturation remained unimpaired by AhR activation. Therefore, AhR agonist signaling exerts specific effects on monocyte/DC subset differentiation of myeloid progenitor cells via interfering with lineage-determining transcriptional processes (Fig. 6).

Whether AhR signaling regulates PU.1 gene expression through direct interactions via cis-regulatory DNA elements or via indirect effects remains to be investigated. In support of the first possibility, we found two putative AhR binding XREs at −3297 and +215 when we screened DNA sequences covering 5000 bp upstream and 1000 bp downstream of the transcriptional start site of the PU.1 gene. Interestingly, the murine PU.1 promoter also contains a potential XRE site at (+180). Alternatively, but not mutually exclusive, an indirect regulation of PU.1 expression might be operative involving other crucial regulatory factors like NF-1, Sp1, or Rb (39–41).

VAF347 arrested cell differentiation at a monocytic precursor cell stage both in M-CSF-dependent monocyte differentiation cultures and in LC generation cultures. In the first differentiation model, CD11bhighCD14high monocytes are generated, and VAF347 addition arrested their differentiation at an earlier stage identified as CD14+CD11bdim/neg cells. Similarly, in LC differentiation cultures, VAF347 inhibited the differentiation of CD1a+Langerh+ cells at a CD14dimCD11bhigh monocytic precursor cell stage. This latter inhibitory effect on monocytic LC precursors by VAF347 is in line with our previous demonstration that LCs arise via monocytic intermediates in cultures of CD34+ cells (18, 38). Furthermore, these observations fit to the here-described selective VAF347-mediated proliferation inhibition in monocyte and LC cultures. In comparison, G-CSF-dependent granulocyte cultures remained unimpaired by AhR agonists. Moreover, PKH26 dye dilution experiments revealed that VAF347 exerts a specific anti-proliferative effect on precursors undergoing monocyte or LC lineage differentiation. This latter finding supports that precursors of these lineages are selectively targeted by AhR activation. This is in line with our finding that AhR activation impairs monocyte and LC differentiation at a monocytic precursor cell stage.

Interestingly, in contrast to monocyte/LC differentiation from CD34+ hematopoietic progenitor cells, in vitro generation of CD1a+DCs from blood monocytes in response to GM-CSF plus IL-4 stimulation (i.e., moDC cultures) was not inhibited by AhR activation. This finding confirms our previous observations that VAF347 fails to interfere with the acquisition of immature DC features from monocytes (12). The underlying mechanism explaining differential susceptibility of DCs from progenitor vs monocyte cultures remains to be elucidated. Monocyte to DC differentiation can be observed in the absence of cell cycling (42), whereas in this study we describe that VAF347 impairs the proliferation of cells in M-CSF-dependent monocyte or TGF-β1-dependent LC cultures. Therefore, the inhibitory effect of VAF347 on the monocyte/DC system seems to be restricted to proliferative monocyte precursor cells.

We demonstrated in this study that AhR activation fails to directly affect LC maturation, indicating that epithelial DCs are not directly impaired by VAF347. Impaired regeneration of immature DCs might be an important mechanism by which AhR ligands interfere with T cell-mediated pathogenic immune responses. This is in line with the observations that VAF347 fails to be effective when applied only during local rechallenge in an allergic asthma model (12). In this model, VAF347-mediated suppression of allergic asthma was only seen when the compound was added during the sensitization phase. Similarly, in the allotransplantation model (13), compound treatment had to be applied for 30 days to result in optimal immunosuppression. A recent study generated tolerogenic DCs by adding VAF347 at culture onset to murine DC cultures initiated by bone marrow cells (13). In this study, VAF347 was present throughout DC differentiation from proliferating progenitors. We similarly added VAF347 at culture onset to LC generation cultures of CD34+ cells. Because the addition of VAF347 to differentiated LCs did not impair LC maturation, it is interesting to speculate that the main modulatory effect of VAF347 on DCs might be due to impairment of DC differentiation. Whether these cells generated in the presence of VAF347 in the human system might share functional characteristics with murine DCs generated...
in this previous study from bone marrow cells remains to be analyzed. In our study, the profound arrest of LC development in response to AhR agonist signaling was found using a human clinically relevant serum-free DC generation protocol. Therefore, for studies toward a future clinical application of AhR agonists, serum-free LC generation cultures of CD34+ hematopoietic progenitor cells might be supplemented with VAF347 and tested for their capacity to modulate T cell mediated immune responses.

Acknowledgments
We thank the collaborating nurses and doctors from the obstetric departments at Kaiser Franz Josef Hospital and the General Hospital, Vienna. We are grateful to T. Graf for providing pPU3.3-TK-luc. We thank S. Taschner, A. Joergl, and F. Gobel for fruitful discussions and help in experimental work. We thank E. Kriehuber and D. Maurer for providing lyses of ex vivo-isolated epidermal LCs. Furthermore, we thank V. Lekska for fruitful discussion and J. Mihaly for providing PCR primers. Finally, J. Stöckl and J. C. Kagan are acknowledged for reviewing the manuscript.

Disclosures
The authors have no financial conflict of interest.

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13. Stöckl and J. C. Kagan are acknowledged for reviewing the manuscript.

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