Langerhans Cell Maturation and Contact Hypersensitivity Are Impaired in Aryl Hydrocarbon Receptor-Null Mice[1]

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Langerhans cells (LC) are professional APCs of the epidermis. Recently, it was suggested that they are tolerogenic and control adverse immune reactions, including against low molecular mass chemicals. The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, is involved in low molecular mass chemical metabolism and cell differentiation. Growing evidence suggests a role for the AhR in the immune system, for example, by influencing dendritic cell and T cell differentiation. We found that the AhR and its repressor AhRR are expressed in LC of C57BL/6 mice. LC, unexpectedly, did not respond to a strong AhR agonist with induction of transcripts of xenobiotic metabolizing enzymes. To test for a physiological role of the AhR in LC, we investigated how AhR deficiency affects LC. We found that AhR-deficient LC were impaired in maturation; they remained smaller and less granular, did not up-regulate expression of costimulatory molecules CD40, CD80, and CD24a during in vitro maturation, and their phagocytic capacity was higher. Interestingly, the mRNA expression of tolerogenic *Ido* was severely decreased in AhR-deficient LC, and enzyme activity could not be induced in AhR-deficient bone marrow-derived dendritic cells. GM-CSF, needed for LC maturation, was secreted in significantly lower amounts by AhR-deficient epidermal cells. Congruent with this impaired maturity and capacity to mature, mice mounted significantly weaker contact hypersensitivity against FITC. Our data suggest that the AhR is involved in LC maturation, both cell autonomously and through bystander cells. At the same time, the AhR might be part of the risk strategy of LC against unwanted immune activation by potential skin allergens. ***The Journal of Immunology,*** **2009, 182: 6709–6717.**

Langerhans cells (LC),[3] which make up 1–3% of the epidermal cells, are the main APCs in the skin, where they act as sentinels. As dendritic cells (DC), they acquire, process, and subsequently present both foreign and self-Ags to T lymphocytes in lymphoid organs, leading to either the induction of immune responses or tolerance. The skin is an important barrier to the environment and is in constant contact with pathogens and biological and anthropogenic substances. Low molecular mass chemicals and their intracellular metabolites are usually not recognized by T cells. However, if these chemicals are capable of binding to proteins they may become part of a presented peptide and thus form neoantigens. It is well known that highly protein-reactive chemicals can lead to sensitization after dermal contact, resulting in contact hypersensitivity (CHS) (1).

Inert low molecular mass chemicals can become protein-reactive as a result of degradation by detoxifying enzymes of the xenobiotic metabolizing enzyme system, such as cytochrome P450 isozymes, or NAD(P)H:quinone oxidoreductase (NQO1). The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that belongs to a highly conserved family of basic helix-loop-helix proteins, is known to be a sensor of certain low molecular mass chemicals and is able to induce genes of the xenobiotic metabolizing enzyme system, which metabolize these compounds in turn. Upon ligand binding the AhR translocates from the cytosol to the nucleus where it forms a heterodimer with the AhR nuclear translocator (ARNT). This dimer binds to xenobiotic-responsive elements present in many promoters and leads to transcription of a wide range of genes not only for xenobiotic metabolism but also genes involved in regulation of cell differentiation, proliferation, and activation (reviewed in Ref. 2). The AhR pathway is regulated by the AhR repressor (AhRR), a target gene of the AhR in a negative feedback loop. The AhRR competes with AhR for the binding site of ARNT and forms a transcriptionally inactive complex (3).

It is known that the skin performs active metabolic functions, including xenobiotic metabolism. For instance, cytochrome P450 monooxygenase CYP1A1, an important xenobiotic metabolizing enzyme and most prominent target gene of the AhR, is expressed in the epidermis, more precisely in keratinocytes (4). The skin is also a target organ of the deleterious effects of toxic AhR activation by environmental pollutants, causing human skin diseases such as chloracne, hyperkeratosis, and photosensitivity. These diseases occurred after human exposure to the AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (5). In the immune system, abnormalities in development and immunosuppression were observed, either after overactivation (i.e., TCDD exposure) or in the absence of the AhR in gene-deficient mouse strains (6–10). Recent studies have pointed to dendritic cells (DC) as the direct target of TCDD and congeners, which then mediate the immunosuppressive effects on T and B cells (11–13).

It appears self-evident that immune responses against low molecular mass chemicals must be under strict control in the skin to

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Received for publication November 5, 2007. Accepted for publication March 19, 2009.

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[1] This research was supported through the German Bundesministerium für Umwelt (Grant BMU.B2).

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[3] Abbreviations used in this paper: LC, Langerhans cell; AhR, aryl hydrocarbon receptor; AhRR, AhR repressor; ARNT, AhR nuclear translocator; BM, bone marrow; BMDC, bone marrow-derived dendritic cell; CHS, contact hypersensitivity; DC, dendritic cell; NQO1, NAD(P)H:quinone oxidoreductase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WT, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0713344

suppress frequent unwanted allergic reactions. Recently, in a shift of paradigm, novel findings have suggested that LC, beyond initiating CHS (14), have regulatory and tolerogenic functions (15). Furthermore, LC were shown to be unable to elicit T cell responses to certain viral Ags (16, 17). The underlying mechanisms are not known.

We here study parameters of LC maturation and biology in $AhR^{-/-}$ mice. Congruent with the observed functional and phenotypic impairments, we find a failure to generate CHS. Our data point to a critical role of the AhR in normal LC maturation.

Materials and Methods

Experimental animals

C57BL/6 mice were purchased from Janvier Laboratories, and $AhR^{-/-}$ mice (bred onto the C57BL/6 background) were from Charles River Laboratories. These mice lack a functional AhR, as the bHLH domain is deleted (18). Mice were bred and housed in our own animal facility under specific pathogen-free conditions. Six- to 12-wk-old female mice received a subtoxic dose of 10 μg TCDD/kg body weight i.p. (LGC Standards) initially dissolved in DMSO and then diluted in corn oil (Sigma-Aldrich) or vehicle (DMSO in corn oil) alone. Mice were sacrificed by CO_2 asphyxiation for removal of organs. The experiments have been reviewed and permitted in accordance with relevant German animal welfare laws.

Cell preparations

Epidermal cell suspensions were prepared from ears and dorsal skin of C57BL/6 and $AhR^{-/-}$ mice as described elsewhere with some modifications (19). Briefly, skin was rinsed with 70% alcohol; ears were split with the aid of forceps and placed, dermal side down, on a 0.25% trypsin/PBS solution for 2 h at 37°C. In the case of dorsal skin, the s.c. fat was scraped off before placement on trypsin. Epidermal sheets were then peeled from the underlying dermis and floated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L -glutamine, 5×10^{-5} M 2-ME, 0.15% sodium hydrogencarbonate, 1 mM sodium pyruvate, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (PAA Laboratories), hereafter referred to as complete medium. Cells were released by breaking up sheets with forceps and vigorous pipetting. The resulting cell suspension consisted of ~90% keratinocytes and 1–3% LC. These cells are referred to as "epidermal cells" in the text.

For cell culturing of epidermal cells, an additional digestion with DNase I (15 min, 130 U/ml; Invitrogen) was included. The resulting single-cell suspension either was used directly for sorting or was cultured in complete medium for various times at a density of 1.5×10^6 cells/ml in 6-well plates. For sorting, epidermal cells were centrifuged on dense BSA or OptiPrep (Sigma-Aldrich) as described (19), or according to the instructions of the manufacturer. The low-density fraction contained 20–35% LC, which were enriched by FACS sorting of vital MHC-II^+ cells using a FACSCalibur (BD Biosciences) to a purity of >95%. Sort purity was verified by reanalysis of samples.

CHS assay

Mice were sensitized by painting 200 μl of 0.05% (w/v) FITC on their shaved backs. FITC was dissolved in a 1:1 (v/v) mixture of acetone/dibutylphtalate. For negative controls, mice were painted with solvent only. Five days later the ear thickness of both ears was measured as reference value and 20 μl of 0.03% FITC was applied to both ears. Twenty-four hours after the challenge ear swelling on both ears was measured with a spring-loaded micrometer (Mitutoyo). CHS was determined as the amount of ear thickness after challenge compared with ear thickness before challenge. The experiment was repeated twice.

Abs, flow cytometry, and Western blotting

The following anti-mouse Abs were obtained from BD Pharmingen: anti-CD11c (clone N418), anti-CD16/32 (clone 2.4G2), anti-CD24A (clone M1/69), anti-CD40 (clone 3/23), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), and anti-I-A/I-E (clone 2G9). Cells were preincubated with unconjugated CD16/32 before staining to block Fc receptors (except samples, where CD16/32 expression was analyzed). All stainings were performed for 15 min at 4°C. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

Western blotting of sorted LC, bone marrow-derived DC (BMDC) and various tissues was done with standard procedures, using BIOMOL (catalog no. SA210) polyclonal rabbit anti-mouse AhR Ab and goat anti-rabbit Ab to develop. Loading was controlled with anti-GAPDH Ab clone 6C5 (Acris), and bands were densitometrically quantified.

Gene expression analysis with microarray

Total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. For microarray analysis mRNA was amplified before chip hybridization using the MessageAmp kit of Ambion. RNA was biotinylated (Enzo BioArray HighYield RNA transcript labeling kit from Affymetrix) and purified. RNA was hybridized to MOE430A gene chips (Affymetrix). The resulting *.chp files were analyzed with the bioconductor affy package using the RMA (robust microarray analysis) algorithm. Two independent experiments were performed. A 2-fold expression difference was chosen as cut-off. Internal controls on the chip excluded a faulty preparation of the chips. The Gene Expression Omnibus accession no. is GSE9506 (www.ncbi.nlm.nih.gov/geo/).

Reverse transcription

For cDNA synthesis, total RNA was treated with 1 U DNase I/0.5 μg RNA (15 min, room temperature). The reaction was stopped with 2 mM EDTA for 10 min at 65°C. RNA (0.5 μg) was incubated in 10 μl with 1 μg of oligonucleotide $pd(T)_{16}$ primer for 5 min at 60°C. RNA was reverse transcribed in a final volume of 40 μl containing 1× RT buffer, 10 mM DTT, 1 mM dNTP, 80 U of RNaseOUT RNase inhibitor, and 400 U of murine leukemia virus reverse transcriptase. Reactions were conducted for 60 min at 37°C and were inactivated at 70°C for 10 min. All reagents were purchased from Invitrogen.

Polymerase chain reaction

Real-time PCR was performed on a Rotor-Gene RG 3000 (LTF Labortechnik) in 15 μl of final volume, containing 7.5 μl of SensiMixPlus SYBR PCR kit (Quantace), 1 μM each primer, 1 μl of cDNA, and RNase-free water. Amplification conditions were 45 cycles of 15 s at 94°C for denaturation; 20 s at 55°C (*AhRR*, *Aldh3a1*, *Cyp1A1*, *Nqo1*), 56°C (*AhR*), and 58°C (*Cyp1A2*, *Cyp1B1*, *Ido*), respectively, for primer annealing; 30 s at 72°C for elongation; and 2 s at 72°C for fluorescence detection.

Sequences of PCR primers were as follows (forward and reverse, respectively): *AhR*, 5′-AGG ACC AAA CAC AAG CTA GA-3′ and 5′-TGG AGA TCT CGT ACA ACA CA-3′; *AhRR*, 5′-GCC AAT GCT GTC TAA TGA AG-3′ and 5′-AAC AGA GCA CCA AGA AAA CA-3′; *Aldh3a1*, 5′-CCC CTG GCA CTC TAT GTG TT-3′ and 5′-CTC TTG CCT GGT GAG GTC TC-3′; Cyp1A1, 5′-TCC TTG CAT GTC CAT GTT TC-3′ and 5′-TGC ATA AGC AAA ATA CAG TCC A-3′; *Cyp1A2*, 5′-CTT TGA CAC AGT CAC CAC AG-3′ and 5′-CTT CTC ATC ATG GTT GAC CT-3′; *Cyp1B1*, 5′-GAC CCG GAT GTT TTG TGA AT-3′ and 5′-CAT GGT GAG CAG CAA AAG AA-3′; *Ido*, 5′-GAG TCT TGA TGT CCT TCT GG-3′ and 5′-CTA CTA TTG CGA GGT GGA AC-3′; *Nqo1*, 5′-GGA CAT GAA CGT CAT TCT CT-3′ and 5′-TTC TTC TTC TGC TCC TCT TG-3′; *Rps6*, 5′-ATT CCT GGA CTG ACA GAC AC-3′ and 5′-GTT CTT CTT AGT GCG TTG CT-3′.

Expression levels were calibrated to the expression of *Rps6* as housekeeping gene in the same sample.

Analysis of *AhRR* expression in heart, brain, and LC was performed on a Trio thermocycler (Biometra). The reaction volume of 50 μl contained 2 μl of cDNA (see above), 2.5 U of *Taq*DNA polymerase, 5 μl of 10× PCR buffer (NatuTec), 0.2 mM concentration of dNTPs (NatuTec), and 0.4 μM concentration of each primer (Operon Biotechnologies). Amplifications were conducted for 5 min at 94°C, followed by 35 cycles for *AhRR* and 30 cycles for *Rps6* of 1 min at 94°C for denaturation, 1 min at 54°C (*AhRR*) and 56°C (*Rps6*), respectively, for primer annealing, 1 min at 72°C for elongation, and 10 min at 72°C after the last cycle.

Phagocytosis assay

Freshly isolated epidermal cells from wild-type (WT) and $AhR^{-/-}$ mice were resuspended in PBS/1% BSA and incubated with 0.5 mg/ml FITC-dextran (M_r of 40,000; Sigma-Aldrich) for 45 or 90 min at 4°C or 37°C. Cells were washed three times with ice-cold PBS containing 1% BSA. After staining with allophycocyanin-conjugated anti-I-A/I-E mAb, viable LC were analyzed by flow cytometry. The percentage of MHC-II^+/$FITC^+$ living cells was determined.

IDO enzyme activity measurements

IDO activity was measured in DC generated from GM-CSF-stimulated bone marrow and treated on day 6 with either 100 U/ml IFN-γ (R&D Systems) or 100 ng/ml LPS. Twenty-four hours later, supernatants were collected. More than 80% of the nonadherent cells expressed CD11c. Generation of kynurenine indicative of IDO activity was measured in culture

supernatants as described (20) with a colorimetric assay. The amount of kynurenine was calculated using a serially diluted kynurenine standard (Sigma-Aldrich).

GM-CSF measurement

Epidermal cells were isolated as described above and seeded at 10^6 cells/ml. Cells were cultured for 48 h. GM-CSF concentrations in the supernatants were determined by ELISA (eBioscience) according to the manufacturer's instructions.

Cultivation of epidermal cells with GM-CSF

Epidermal cells were seeded at a concentration of 10^6 cells/ml and cultured with 10 pg or 100 pg/ml GM-CSF. After 48 h, cells were harvested and analyzed by flow cytometry.

Statistical analysis

The paired Student's *t* test or Mann-Whitney *U* test were used to assess statistical significance of data. Values of $p < 0.05$ were considered as significant.

Results

AhR expression and responsiveness in LC

The AhR is expressed at varying levels in many tissues and cell types, including lymphoid organs like thymus and spleen. Murine keratinocytes show a pattern of increased AhR expression correlated to differentiation, and AhR expression in dermal fibroblasts has been described as well (21, 22). Here, we demonstrate for the first time AhR expression in LC. Easily detectable by RT-PCR and Western blotting, LC expressed AhR protein at much higher levels than liver or thymus (Fig. 1*A*). Epidermal cells (which consist of >90% of keratinocytes) and in vitro BMDC also expressed AhR. Very little AhR protein was detected in bone marrow and brain. Thus, protein levels reflected RNA levels (compare Fig. 1*A* and supplemental Fig. 1).[4]

We analyzed whether typical AhR target genes are induced in vivo in skin cells and LC after systemic exposure to the AhR-activating ligand TCDD and found that *Cyp1A1*, *Cyp1B1*, *Nqo1*, and *Aldh3a1* mRNA were inducible in epidermal cells, but not in highly pure LC of TCDD-exposed mice (Fig. 1*B*). A global gene expression profile analysis (Affymetrix) of LC purified to >95% by density gradient centrifugation and FACS sorting of MHC-II$^+$ cells (23) yielded no TCDD-inducible transcription, even at a moderate 2-fold expression difference. We could not detect any change in the expression of the 22,500 genes presented on the chip in treated vs control groups (data not shown; Gene Expression Omnibus accession no. GSE9506, www.ncbi.nlm.nih.gov/geo/). Thus, in contrast to many other cell types, LC appeared inert against TCDD-mediated gene induction, albeit they expressed the AhR at high levels.

Expression of the AhRR in epidermal LC of WT and Ahr$^{-/-}$ mice

The AhRR is differentially expressed in tissues. Generally, in AhR$^{-/-}$ mice the AhRR mRNA content is two to three orders of magnitude lower than in WT mice (24). As shown in Fig. 1, WT LC expressed the AhR, and the AhRR. AhRR transcripts were detectable in LC by RT-PCR, and they could be further induced after TCDD exposure. AhRR expression was lower, but not absent, in LC of AhR-deficient mice in comparison to WT counterparts (Fig. 2*A*, compare *lanes 3* and *4*). AhR signaling is regulated by the AhRR in a negative feedback fashion. The AhRR competes with the AhR in dimerization with ARNT to form a transcriptionally inactive complex (3). Note that AhRR expression was constitutively high in WT LC (Fig. 1*Bf*). Brain and heart are the organs

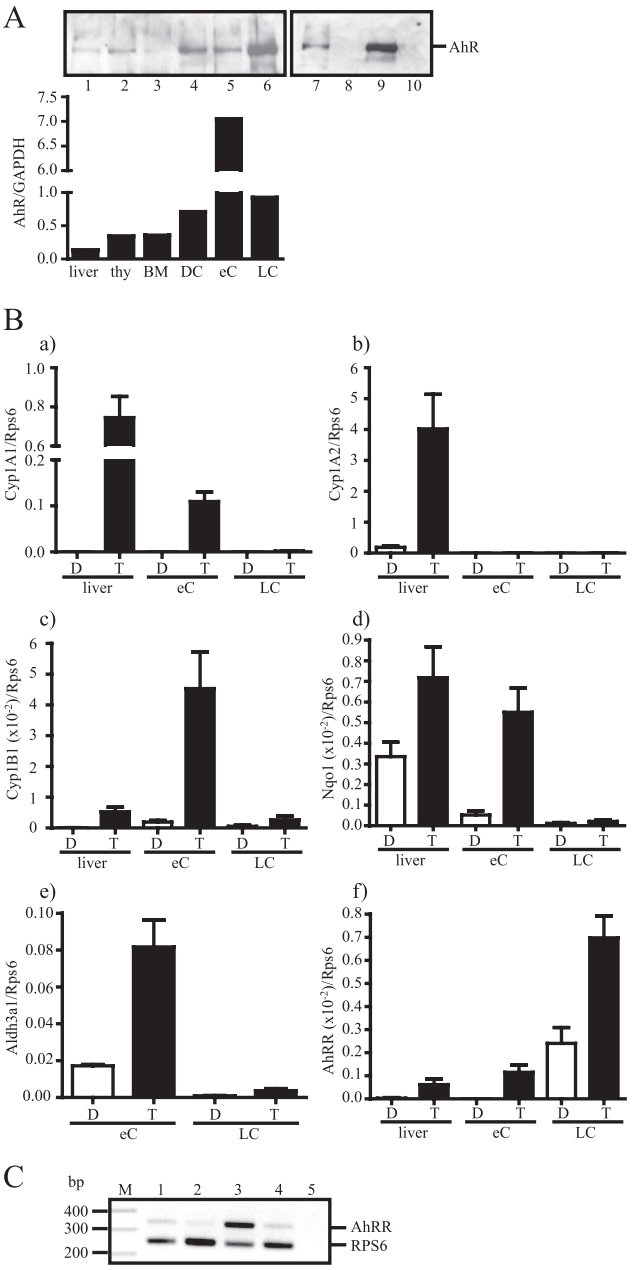

FIGURE 1. AhR expression and inducibility of xenobiotic metabolizing enzymes in epidermal cells and LC. *A*, Western Blot showing expression of AhR in various cell subsets and tissues. Protein was prepared from organs or sorted cells and protein lysates were blotted with polyclonal rabbit anti-AhR (BIOMOL SA210). *Lanes 1–6*, Protein (800 ng) from WT mice was loaded: (*1*) liver, (*2*) thymus, (*3*) bone marrow, (*4*) BMDC (day 6 of culture, 80% CD11c$^+$ cells) (*5*) epidermal cells, and (*6*) Langerhans cells (sorted to >97% purity from mouse back skin); *lanes 7–10* show a blot with 2 μg of protein from WT and AhR$^{-/-}$ mice: (*7*) liver, WT mice; (*8*) liver, AhR$^{-/-}$mice; (*9*) BMDC WT mice; and (*10*) BMDC AhR$^{-/-}$ mice. Transfer was controlled with Ponceau staining and loading with GAPDH Ab. Bands were densitometrically evaluated. The bar graph shows expression after calibration to GAPDH levels on the blot. *B*, C57BL/6 mice were injected with 10 μg/kg TCDD or the solvent DMSO alone. Relative expression levels of xenobiotic metabolizing enzymes and of AhRR was measured by real-time PCR from epidermal cells and LC sorted to >98% purity. For comparison, liver is included as an organ with known high metabolic activity. D, control animals, treated with solvent DMSO; T, TCDD-treated animals. *C*, Expression levels of AhRR in LC. mRNA was prepared, reverse transcribed, and amplified by PCR for *AhRR* and the *Rps6* housekeeping gene: *Lanes 1–4*: (*1*) heart, (*2*) brain, (*3*) LC isolated from WT mice, and (*4*) LC isolated from AhR$^{-/-}$ mice. S, 100-bp marker; N, negative control without DNA in PCR. The image is representative for three independent experiments.

[4] The online version of this article contains supplemental material.

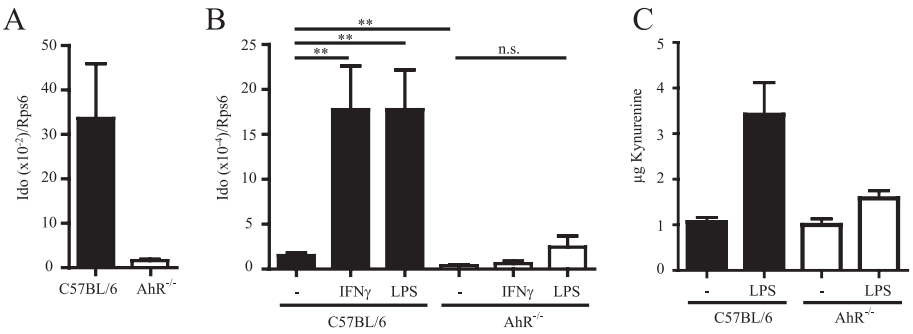

FIGURE 2. IDO expression and regulation in AhR$^{-/-}$ LC and BM-derived DC. *A*, LC were sorted by FACS to >95% purity from epidermal cells (cultivated from ear epidermal sheets for 16 h); RNA was isolated, amplified, reverse transcribed, and used for RT-PCR. Shown is the relative expression for C57BL/6 LC to AhR$^{-/-}$ LC, after calibration with housekeeping gene *Rps6*. The experiment was repeated twice. Similar results were obtained with unamplified RNA. *B*, Bone marrow was cultivated for 6 days in the presence of GM-CSF to generate DC, and then LPS (100 ng/ml) or IFN-γ (100 U/ml) was added for 24 h to induce IDO. RT-PCR was performed. Shown is the *Ido* expression relative to the housekeeping gene *Rps6*. Data are from five independent experiments. Significance was tested with student's *t* test. **, $p < 0.001$; ns, not significant. *C*, Generation of kynurenine indicative of IDO activity was assessed in culture supernatants of BMDC, stimulated for an additional 72 h with LPS and L -tryptophan as described (20). Kynurenine was quantified with a colorimetric assay against a kynurenine standard (Sigma-Aldrich). Data are from two to three individual mice.

with the highest constitutive expression of AhRR transcripts known so far (24), and AhRR expression in WT LC was even higher than in those tissues (Fig. 1*C*).

Transcription profiles of WT and AhR$^{-/-}$ LC differ

LC were sorted out of ear epidermal cell cultures to >95% purity. RNA from LC from five mice was pooled and hybridized to an Affymetrix chip.

A total of 127 genes expressed differentially (threshold 2×) between the two genotypes. Table I lists differentially expressed genes of the immune system. Approximately 20 transcripts of the immune system were more abundant in AhR$^{-/-}$ LC, including CD36 and MHC-II, suggesting that the AhR might contribute to their transcriptional regulation. More genes were down-regulated in AhR$^{-/-}$ LC; that is, their normal expression level is AhR-dependent. Confirming the PCR results, the high constitutive expression of AhRR was lost in AhR$^{-/-}$ LC. The most highly deregulated gene was *Ido*, with a lower transcript abundance in AhR$^{-/-}$ compared with WT LC. The decrease was strong and significant, as confirmed by quantitative RT-PCR of LC cultivated for 20 h (Fig. 2*A*). As expected from reports in the literature, *Ido* expression was absent in WT LC isolated directly from skin without prior cultivation (data not shown). We tested *Ido* mRNA up-regulation by the known inducers LPS or IFN-γ in BMDC. As shown above, these DC express high level of AhR, similar to LC. Absence of AhR almost completely prevented normal *Ido* up-regulation by IFN-γ. LPS led to a slight, but not significant, induction of *Ido* mRNA (Fig. 2*B*). Also,

Table I. *Differentially expressed immune-related genes in AhR$^{-/-}$ LC*[a]

Probeset ID	Gene Name[b]	Symbol	Fold Damage
Lower transcription in AhR$^{-/-}$ LC			
1420437_at	Indoleamine-pyrrole 2,3-dioxygenase	Indo[c]	8.82
1420796_at	Aryl-hydrocarbon receptor repressor	Ahrr	8.63
1416811_s_at	Cytotoxic T lymphocyte-associated protein 2	Ctla2a///Ctla2b	3.48
1424836_a_at	CLIP associating protein 2	Clasp2	3.37
1423489_at	Monocyte to macrophage differentiation-associated	Mmd	2.16
1419872_at	CDF1 receptor	Csf1r	2.07
1427418_a_at	Hypoxia inducible factor 1, α subunit	Hif1a	2.03
Higher transcription in AhR$^{-/-}$LC			
1417292_at	IFN-γ-inducible protein 47	Ifi47	2.00
1417292_at	C-type lectin domain family 4, member d	Clec4d	2.09
1420804_s_at	Complement component 1, q subcomponent, α polypeptide	C1qa	2.14
1417381_at	IFN-γ-inducible protein 30	Ifi30	2.15
1422476_at	CD68 Ag	Cd68	2.17
1434366_x_at	IL-1β	Il1b	2.29
1449399_a_at	IFN-activated gene 205	Ifi205	2.37
1450648_s_at	CD36 Ag	Cd36	2.40
1450883_a_at	Macrophage-expressed gene 1	Mpeg1	2.42
1435290_x_at	Complement component 1, q subcomponent, β polypeptide	C1qb	2.48
1437726_x_at	Histocompatibility 2, class II Ag E β	H2-Eb1	2.59
1425477_x_at	CD274 Ab	Cd274	2.69
1452231_x_at	Histocompatibility 2, class II Ag A, β1	H2-Ab1	2.90
1451721_a_at	Chemokine (C-X-C motif) ligand 4	Cxcl4, Pf4	3.11
1448995_at	IFN-activated gene 203	Ifi203	3.16
1426906_at	Histocompatibility 2, class II Ag A, α///histocompatibility 2, class II Ag E α	H2-Aa///H2-Ea	3.98

[a] LC were isolated from ear epidermal cells by FACS sorting. RNA was prepared, amplified, and hybridized to Affymetrix MOE430A chips. Analysis was done as described in *Materials and Methods*.

[b] Common name and official gene symbol.

[c] Also known as Ido.

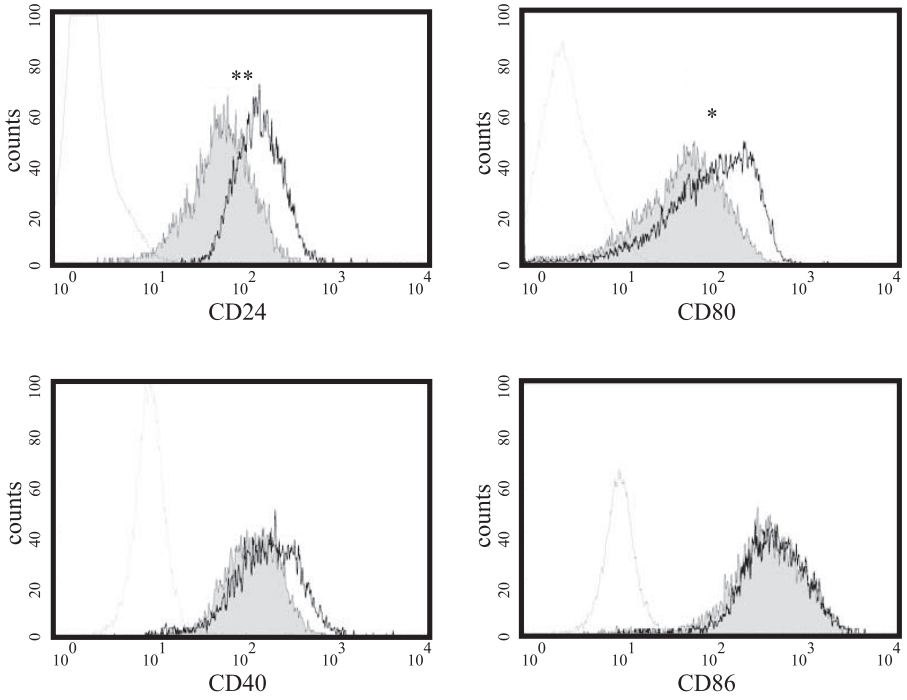

FIGURE 3. Expression of costimulatory molecules on mature LC is dependent on the AhR. WT (white curve) and AhR$^{-/-}$ (gray curve) mice, respectively, are shown. Mice were sacrificed, and epidermal cells were isolated as described in *Materials and Methods* and cultured further for 72 h. Cells were harvested, and LC were enriched over BSA density gradient centrifugation, stained with described Abs, and analyzed by FACS. Dotted line designates isotype control. Shown are representative data from three to four independent experiments. Significances were calculated by Student's *t* test. *, $p < 0.05$.

induction of generation of kynurenine from tryptophan, which is driven by IDO enzymatic activity, was much less in AhR$^{-/-}$ DC (Fig. 2*C*). Thus, AhR is necessary for IDO expression in LC/and DC, and for IDO function in DC.

Impaired maturation competence of LC in AhR$^{-/-}$ mice

Dependent on their differentiation and maturation status, LC express surface proteins necessary for stimulation of T cells (e.g., MHC-II, CD24a, CD40, CD80, and CD86). LC have been shown to mature during ex vivo culture of epidermal cells (19). Therefore, we analyzed expression levels of maturation markers on the cell surface of freshly isolated ("immature") LC and on LC after 3 days of culture ("mature") of epidermal cells from either WT or AhR$^{-/-}$ mice.

Treatment of cells for 10 min with trypsin did not impair Ab staining, indicating that the surface markers described here are not sensitive to the treatment necessary for preparing epidermal sheets/cell suspensions (data not shown).

Fig. 3 shows representative histograms of the expression of costimulatory molecules CD24a, CD40, CD80, and CD86 on mature LC from WT and AhR$^{-/-}$ mice. Table II shows the mean fluorescence intensities of these markers for both mature and immature LC, comparing LC from WT and AhR$^{-/-}$ mice. WT LC of mice injected with TCDD up-regulated CD86 expression levels. As expected, no expression changes of costimulatory molecules by TCDD was detectable in AhR$^{-/-}$ LC (data not shown).

All markers tested, except CD24a, had similar basal expression levels on immature LC. CD24a was expressed significantly lower on immature as well as on mature AhR-deficient LC compared with wild-type LC; in accordance with data in the literature, CD24a expression decreased during maturation (25). As expected, maturation of WT LC during ex vivo culture of epidermal cells led to strong up-regulation of costimulatory molecules CD40, CD80, and CD86. MHC-II was also up-regulated (data not shown). Interestingly, MHC-II surface expression did not differ significantly between LC from WT and AhR$^{-/-}$ mice (supplemental Fig. 2).

Qualitatively, LC from AhR-deficient mice up-regulated costimulatory molecules after 3 days of maturation in culture as well. Quantitatively, though, in comparison to the WT mice, the increase was significantly lower during maturation for cells of AhR$^{-/-}$ mice (Table II, compare upper and lower row) for CD80. For CD40 a similar trend was seen in three independent experiments, albeit statistical significance was not reached.

Morphology and phagocytic capacity of LC from AhR$^{-/-}$ mice

In addition to the expression of surface markers, we analyzed how the AhR affects size and granularity of LC. It is well known that LC change their morphology during maturation and become larger

Table II. *Influence of absence of functional AhR on LC phenotype in immature vs mature cells*[a]

	CD16/32		CD24		CD40		CD80		CD86	
	Immature	Mature†	Immature	Mature	Immature	Mature†	Immature	Mature†	Immature	Mature†
WT	14[b]	2[c]	156	122	13	188	2	99	13	458
AhR KO	13	2	**60****[c]	**56***	9	142	2	**42***	11	401

[a] Single-cell suspensions were prepared from ear or skin epidermis and were either immediately (Immature) analyzed by FACS or cultivated for 3 days to allow maturation of LC (Mature). See *Material and Methods* for details. Cells were stained for MHC-II and the indicated surface markers. LC were identified and gated by MHC-II expression and analyzed for the indicated surface markers. The cell suspension consisted of mainly keratinocytes, some other epidermal cells, and 1–3% LC. Scatter characteristics were used to exclude dead cells from the analysis. As controls, unstained and isotype control-stained cells were used. Cultivation and stainings were done independently three to five times.

[b] Channel of mean fluorescence (MFI), determined by FACS.

[c] The significance of values between C57BL/6 (WT) mice and AHR$^{-/-}$ mice was calculated by Student's *t* test and is indicated by asterisks and boldface numbers. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$. Values differing significantly between immature and mature LC were also calculated and occur in the columns noted with a dagger (†).

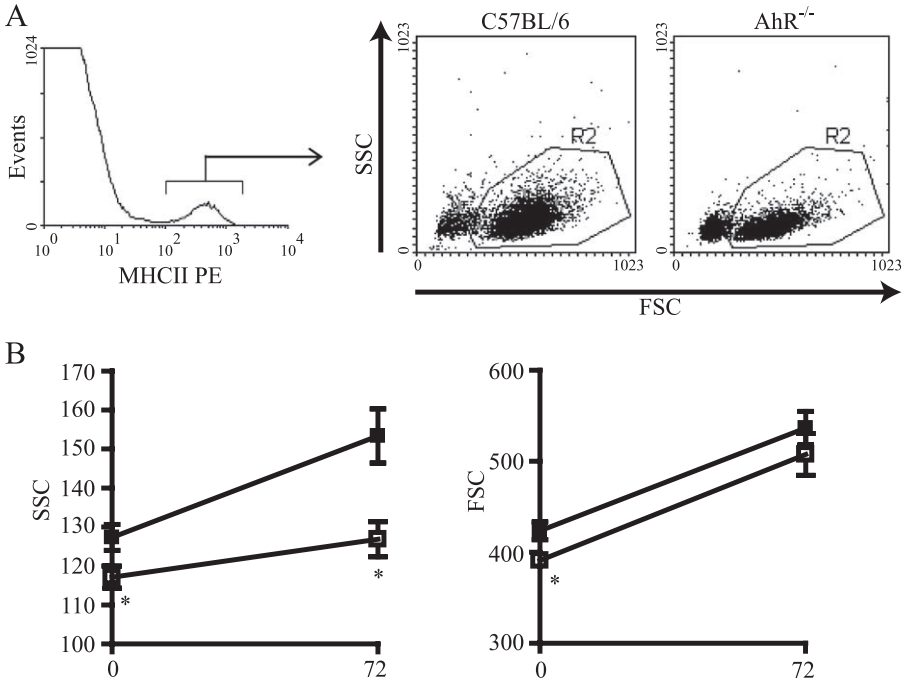

FIGURE 4. LC morphology is dependent on AhR expression. Epidermal cells from WT and AhR$^{-/-}$ mice were isolated and analyzed by FACS. An aliquot of cells was ex vivo cultured for 72 h and analyzed again. *A*, Cells were gated on MHC-II (i.e., for LC), and scatter characteristics were analyzed for WT and AhR$^{-/-}$. *B*, Graphs depicting the change after 72 h of culture. Data are from at least four independent experiments (■, wild-type; □, AhR$^{-/-}$ mice; asterisks indicate significant differences between WT and AhR$^{-/-}$ mice (*, $p < 0.05$)).

and more granular (19). Fluorescence microscopy of AhR$^{-/-}$ epidermal skin sheets did not reveal differences in numbers of LC per mm^2 or striking changes in the ramification of LC dendrites (data not shown). Fig. 4 shows the relative granularity and size of immature and mature MHC-II$^+$ LC from AhR$^{-/-}$ and WT mice. Congruent with the data on the surface marker expression, also the morphology of LC differed between AhR$^{-/-}$ and WT mice after in vitro maturation. In the absence of a functional AhR, immature LC were slightly smaller and remained of lower granularity after maturation. This was not due to the death of MHC-II$^+$ cells, as confirmed by propidium iodide staining. No significant changes of LC from TCDD-treated mice compared with control mice were observed (data not shown).

We analyzed the phagocytic capacity of LC for 45 and 90 min after isolation as a marker for functional immaturity. LC from AhR$^{-/-}$ phagocytosed more at the earlier time point than did LC from WT mice ($p = 0.0536$ at 45 min), suggestive of slower maturation. However, at 90 min this difference had disappeared (Fig. 5). Phagocytosis was abolished completely in both genotypes when LC were cultivated for 24 h (data not shown).

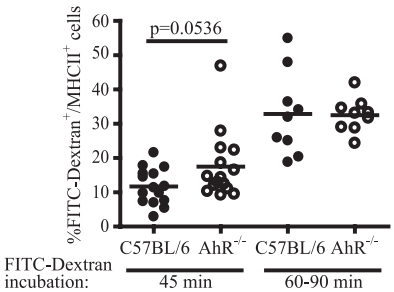

FIGURE 5. Phagocytic capacity upon maturation. Freshly isolated epidermal cells were cultured at 4°C or 37°C with FITC-dextran beads and then stained with anti-MHC-II Abs. Shown is the percentage of FITC$^+$/MHC-II$^+$ cells after 45 min and 90 min of uptake. Each dot represents the result from an individual mouse. Data were analyzed by an unpaired Student's *t* test.

GM-CSF secretion is diminished in epidermal cells of AhR$^{-/-}$ mice

GM-CSF is one of the key cytokines for LC survival and differentiation (26). GM-CSF is produced and secreted by keratinocytes in a paracrine fashion, and GM-CSF plasma levels are known to be enhanced by TCDD (27). Therefore, we analyzed the supernatants of cultured epidermal cells (containing ~90% keratinocytes) from AhR$^{-/-}$ and WT mice by ELISA. Results are shown in Fig. 6*A*. Epidermal cells from WT mice secreted more GM-CSF (mean 40 pg/ml) compared with cells isolated from AhR$^{-/-}$ mice (mean, 18 pg/ml). GM-CSF is pivotal for up-regulation of CD80 on maturing LC. As described above, AhR$^{-/-}$ LC are impaired in up-regulation of costimulatory molecules, including CD80. We cultured epidermal cells from AhR$^{-/-}$ and WT mice in the presence of GM-CSF and measured morphology and CD80 expression. As shown in Fig. 6*B*, addition of 10 or 100 pg/ml GM-CSF to cultures

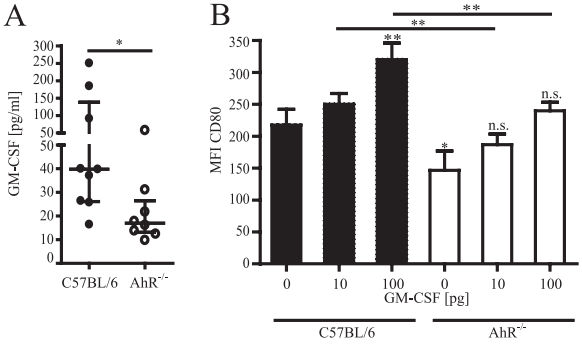

FIGURE 6. GM-CSF-production in AhR$^{-/-}$ epidermal cells. Epidermal cells were prepared from back skin and cultured for 48 h. *A*, Supernatants were tested for GM-CSF content by ELISA. *B*, GM-CSF was added to epidermal cells from AhR$^{-/-}$ and C57BL/6 mice, and cells were cultured for 48 h. The mean fluorescence index (MFI) of CD80 was determined on LC identified and gated by their MHC-II expression. Asterisks on the bars indicate significance against untreated WT cells (*, $p < 0.05$; **, $p < 0.0001$; ns, not significant).

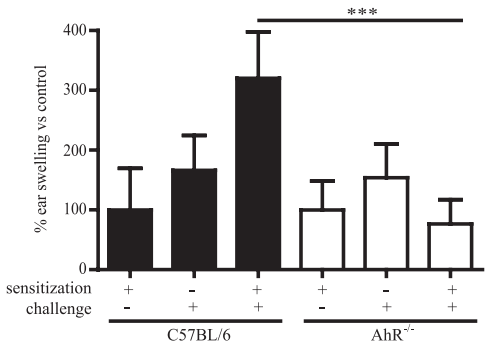

FIGURE 7. Contact hypersensitivity against FITC in AhR$^{-/-}$ mice compared with WT mice. Mice were sensitized and challenged as described in *Materials and Methods*. Ear thickness was measured and the change between both genotypes recorded. The experiment was repeated twice with similar results. Each treatment group comprised five to seven mice. Data were analyzed with Student's *t* test (***, $p < 0.0001$).

brought back CD80 expression of AhR$^{-/-}$ LC to the expression level of WT LC. This concentration is in the range of the GM-CSF secreted by WT epidermal cells (see Fig. 6*A*). Additional GM-SCF induced CD80 expression in WT LC as well, at both 10 and 100 pg/ml to levels that were significantly higher than in AhR$^{-/-}$ LC ($p = 0.0017$ and 0.0016, respectively). Addition of GM-CSF to epidermal cell cultures did not change the low granularity and size.

CHS is impaired in AhR$^{-/-}$ mice

CHS is a functional assay for competent immune responses against Ag entering via the skin. We tested whether CHS is normal or impaired in AhR$^{-/-}$ mice using the fluorescent low molecular mass chemical FITC. AhR$^{-/-}$ mice mounted a lower CHS response (Fig. 7): ear thickness of WT mice was significantly higher ($p < 0.0001$). FITC^{+} DC could be detected in draining lymph nodes of sensitized mice of both strains, indicating that migration is not abrogated (supplemental Fig. 3).

Discussion

Intrinsic and induced cell differentiation and the cellular response to endogenous and exogenous signals are hallmarks of the immune system in fighting pathogens. The AhR as an externally triggered latent transcription factor is strikingly abundant in several immune tissues. mRNA abundance equals or surpasses that of the liver (28), where the main function of the AhR is thought to be xenobiotic metabolic degradation. AhR mRNA expression appears to correlate with the AhR's ligand-induced transcription factor activity. We found a good correlation between mRNA and protein levels for AhR. Also, published interspecies data indicate a reasonable correlation between AhR mRNA and protein levels (29, 28, 30). Growing knowledge suggests that the AhR signaling pathway is relevant in the immune system, used in differentiation and function of immune cells, and links environmental triggers to the immune system (9, 10, 31, 32).

We show here for the first time that primary murine LC express AhR mRNA and protein. Indeed, expression was higher than in other immune cells, and higher than in liver. In an extension of data reported in the literature, AhR expression was high in keratinocytes as well. We analyzed the effects of AhR overactivation and AhR deficiency in LC and epidermal cells. To our knowledge, only one study addressed the effects of AhR activation on LC until now. In 1989, Puhvel et al. (33) investigated density and morphology of LC in HRS/J mice, a murine model for skin effects of TCDD. They reported that LC from hairless mice were smaller and had fewer dendritic protusions than did controls. Extending these early studies, our data are suggestive of a role for the AhR in the maturation of LC.

Immature and semimature LC from mice isolated after exposure to the strong and persistent ligand TCDD had not up-regulated or down-regulated genes for enzymes of the xenobiotic response, which is very unusual for AhR-containing tissues (31, 34). Indeed, they appeared inert to global transcriptome changes. This intriguing finding might be due to either a strong inhibitory feedback loop by the highly expressed AhR repressor in LC (35), or ligand-dependent outcomes of transcriptional changes, as has been suggested for other cell types (28). Additional experiments could test the hypothesis that LC might control xenobiotic metabolism as a means of reducing the risk of pro-hapten to hapten generation from low molecular mass metabolites (28, 36). Several authors have suggested functions of the AhR beyond its ligand-activated transcription factor status, for instance by direct contact and interaction with NF-κB (38, reviewed in Ref. 39). It is possible that beyond ligand- (TCDD-) driven transcriptional activity, nonligand-dependent activities of the AhR are relevant in LC, such as in maturation and function.

As the AhR might induce cell differentiation, we analyzed immature and mature LC from AhR-deficient mice in epidermal cell cultures. Three relevant parameters of LC maturation were analyzed, namely up-regulation of costimulatory molecules, size and scatter characteristics, and phagocytic capacity. Our data provide evidence that in the absence of the AhR in epidermal cells LC maturation is impaired. Interestingly, the number of LC per mm^2 was unaffected in skin.

Note that AhR deficiency led to low CD24a expression in LC, while in splenic DC AhR overactivation induced CD24a (11). A lower constitutive CD24a expression in AhR-deficient mice compared with WT mice could reflect the presence of an endogenous ligand (which would drive AhR activity in normal mice), as already suggested above.

Up-regulation of costimulatory molecules and morphological changes are critical features of LC maturation and their function as potent APCs. In particular, the expression of CD24a and CD80, both significantly low in AhR-deficient LC, might reduce their capacity to stimulate Th1 responses. Considering that splenic lymphocytes from AhR$^{-/-}$ mice produced more IFN-γ and IL-12 after OVA immunization than those from AhR$^{+/+}$ mice, this might be surprising. Note, however, that we look at other cells (i.e., LC) and at a "naive" situation. Moreover, allergic sensitization led to a higher IL-5 production and increased IgE titer in AhR$^{-/-}$ mice compared with AhR$^{+/+}$ mice (40, 41), suggestive of AhR as a driver toward Th2. Several lines of evidence suggest a role for the AhR in thymocyte and T cell differentiation (10, 37, 42, 43), and a number of cytokines can be regulated by AhR signaling, including IL-2 in T cells and TNF-α, and IL-1β in keratinocytes (44, 45). More data are needed to clarify the involvement of AhR in cytokine balance.

Maturation of LC may be influenced by signals from surrounding keratinocytes (which are affected by AhR absence as well). It is therefore not too surprising that epidermal cells secreted less GM-CSF in AhR-deficient cultures, in particular as the gene has four putative xenobiotic-responsive elements in its promoter (46). GM-CSF is relevant for LC maturation, that is, CD80 expression levels (47). LC themselves cannot survive unless they are either surrounded by keratinocytes or GM-CSF is added to cultures (26). GM-CSF addition to WT LC in culture even enhanced CD80 expression levels. The CD80 phenotype of AhR$^{-/-}$ LC could be rescued by GM-CSF and brought back to WT levels. However, the morphological changes persisted. Apparently, other factors

are lacking as well in AhR$^{-/-}$ mice, which warrant further investigation.

AhR$^{-/-}$ do not have a drastic immune phenotype. The animals survive and can reach old age (48). Immune responses against cellular (allogenic P815 tumor cells) and humoral (sheep RBC) model Ags showed normal and competent immune responses (49). Memory response against a protein Ag was reported (8). However, they are more susceptible to infections with *Helicobacter hepaticus*, an opportunistic infection indicating immunodeficiency (50, 51), and to *L.monocytogenes* (52). The underlying cause may be specific defects in the immune system or other general damages resulting from AhR deficiency.

CHS is a skin inflammatory reaction to topically applied haptens mediated by CD8$^+$ T cells primed in skin draining lymph nodes by poorly defined APCs. LC take up Ag, mature, and migrate to the lymph nodes to initiate an immune response or, alternatively, exert tolerogenic functions (53). We show here that CHS is impaired by AhR deficiency. CHS suppression appeared not due to an inability of LC or dermal DC to leave the skin and reach the draining lymph nodes after Ag uptake. Conceivably, the weaker CHS might be due to the low expression of costimulatory molecules and to the persisting immature phenotype we observed. AhR$^{-/-}$ LC were smaller than WT LC, possibly indicating a lower fraction of motile LC destined to migrate to the lymph nodes even in the steady-state (54). Skin-resident cell types, such as keratinocytes and mast cells, and mobile leukocytes, including T lymphocytes and NK cells, actively participate in the CHS reaction. IL-2-producing CD8$^+$ cells and regulatory T cells appear to be crucial in the prevention of contact allergy or in the early termination of the reaction (55, 56). Active AhR signaling can serve as a co-factor in IL-2 production and formation of regulatory T cells (57, 58). Moreover, allergen sensitization induces the development of distinct CD8 T cell subpopulations that produce IL-17 (10, 59), and the AhR acts as a co-factor in IL17-production. More studies are needed to differentiate the contribution of LC vs other cell types in diminished CHS response of AhR$^{-/-}$ mice. This can be done, for example, by LC cell-specific deletion of the AhR. Recently, a study demonstrated that AhR activation affected DC as well as regulatory T cell survival and function, resulting in islet allograft-specific tolerance (60).

An interesting finding was the failure by AhR$^{-/-}$ LC to upregulate *Ido* transcription. Congruently, IDO activity could not be stimulated in BMDC. Expression of IDO upon maturation was recently shown for human LC (10, 61). AhR activation can induce IDO transcription and ensuing enzyme activity in DC (10, 61, 62). Whether IDO expression by LC affects the balance between tolerance and immunity in vivo remains to be determined. It is intriguing to speculate that IDO in LC may serve to keep tryptophan levels low, as tryptophan is a precursor for the UV-induced AhR agonist FICZ (63).

In conclusion, we provide evidence for a role of the AhR in LC maturation and function. These findings contribute to a better understanding of allergic skin immune responses and their medical management.

Acknowledgments

We thank Markus Frericks for expert help and advice regarding microarrays, and Sarah Böddeker for contributions. We thank Alla Velgach and Ninon Krahnke-Schoelzel for technical help, and Michael Nowak, Heike Weighardt, and Irmgard Förster for critical discussions.

Disclosures

The authors have no financial conflicts of interest.

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