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Staphylococcus epidermidis Activates Aryl Hydrocarbon Receptor Signaling in Human Keratinocytes: Implications for Cutaneous Defense

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Cutaneous innate defense · Aryl hydrocarbon receptor · *Staphylococcus epidermidis* · IL-1β

Abstract
Bacterial challenge of keratinocytes with the abundant skin commensal *Staphylococcus epidermidis* induces distinct innate immune responses, but the underlying molecular mechanisms are still emerging. We report that the aryl hydrocarbon receptor (AhR) was activated in human primary keratinocytes infected with *S. epidermidis*, leading to induction of the AhR-responsive gene cytochrome P450 1A1 (CYP1A1). In addition, functional AhR was required for *S. epidermidis*-mediated induction of IL-1β expression in keratinocytes. AhR-dependent gene induction of IL-1β and CYP1A1 was mediated by factor(s) <2 kDa secreted by *S. epidermidis*. Blockade of the AhR in a 3D organotypic skin equivalent infected with *S. epidermidis* attenuated the *S. epidermidis*-induced CYP1A1 and IL-1β expression. Moreover, *S. epidermidis* also induced expression of IL-1α and of the antimicrobial peptide human β-defensin-3 in an AhR-dependent manner in a 3D skin equivalent. An increased outgrowth of *S. epidermidis* on the surface of skin explants treated with a specific AhR inhibitor further indicate a pivotal role of the AhR in mediating an epidermal defense response. Taken together, our data expand the role of the AhR in innate immunity and support a previously unappreciated contribution for the AhR in cutaneous defense.

Introduction

The Gram-positive bacterium *Staphylococcus epidermidis* is an abundant member of the normal human skin microbiota [1]. Its presence on the skin is also associated with a risk of nosocomial *S. epidermidis* infections, especially infections associated with medical devices such as catheter-related infections [2]. However, there is increasing evidence that under normal conditions the presence of *S. epidermidis* on the skin surface seems to be favorable for the host [3] and strengthens cutaneous innate defense [4]. For example, *S. epidermidis* is able to dampen inflammatory processes after skin wounding [5] and it secretes antimicrobial factors, which restrict the growth of other competing microbes [3, 6]. In addition, *S. epidermidis* directly induces the expression of antimicrobial peptides in keratinocytes [7–9] or indirectly via *S. epidermidis*-spe-

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cific T-cell responses [10]. Activation of dendritic cells and keratinocytes by *S. epidermidis* also induces distinct IL-1-mediated immune responses, indicating that IL-1 receptor targeting cytokines such as IL-1α and IL-1β are important mediators of host skin-commensal cross-talk [11, 12]. Since the molecular mechanisms underlying the *S. epidermidis*-mediated activation of IL-1 in keratinocytes remain elusive, the primary aim of this project was to gain more insight into the interaction of *S. epidermidis* and keratinocytes leading to IL-1β production. As a result, we report here a novel role of the aryl hydrocarbon receptor (AhR) in mediating a defense response in keratinocytes exposed to *S. epidermidis*.

The AhR was originally identified as a receptor to polycyclic aromatic hydrocarbons such as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and other environmental pollutants and xenobiotics. It serves as a cytosolic ligand-activated transcription factor which, upon ligand binding, translocates into the nucleus, where it dimerizes with the AhR nuclear translocator (ARNT) followed by binding to specific sites in target gene promoters [13]. Much research in the last 15 years has revealed that the AhR is involved in various physiological aspects such as cell homeostasis, cell proliferation and differentiation, embryogenesis, carcinogenesis, inflammation, and host immunity [13, 14]. Several reports also indicate an important function of the AhR for maintaining the skin barrier [15, 16]. For example, functional AhR seems to be required for normal keratinocyte differentiation [17] and for mouse skin barrier integrity [18]. Moreover, activation of the AhR has been reported to dampen skin inflammation in psoriasis [19, 20] and to improve the skin barrier in atopic dermatitis [21]. Whereas these reports illustrate an important role of the AhR in cutaneous barrier homeostasis, the role of the AhR in cutaneous innate defense is less understood.

Here we provide evidence that *S. epidermidis* activates the AhR in human primary keratinocytes to induce an innate defense response. These data highlight a novel role of the AhR in cutaneous innate defense and show that the AhR is involved in sensing bacteria by keratinocytes.

Materials and Methods

Cell Culture and Stimulation

For stimulation experiments, pools of primary human keratinocytes derived from different donors (Promocell, Heidelberg, Germany) were used at passage 3–5 and cultured in 24-well plates in Keratinocyte Growth Medium 2 (KGM2) plus supplements (Promocell) without antibiotics at 37 °C in a 5% CO_2 atmosphere. *S. epidermidis* skin-derived clinical isolates (identity verified by MALDI-TOF mass spectrometry; MALDI Biotyper, Bruker, Billerica, MA, USA) and *S. epidermidis* ATCC 14990 were maintained on blood agar plates. For stimulation, bacteria were grown overnight at 37 °C in tryptic soy broth, diluted and grown for an additional 3–4 h until the OD_{600} reached at least 0.2. Bacteria were harvested by centrifugation, washed twice with PBS and resuspended in cell culture medium KGM2 without supplements to the appropriate density. Confluent keratinocytes were stimulated with bacteria at a concentration of approximately 5×10^6 colony forming units (CFU) per well. Bacteria were centrifuged on cells at 350 *g* for 5 min followed by incubation at 37 °C for the indicated time periods. For overnight stimulation, the culture medium was removed after 3 h and cells were washed twice with PBS to remove nonadherent bacteria. The cells were then incubated for an additional 17–19 h in culture medium supplemented with 200 μg/mL gentamicin. After incubation, cell supernatant was harvested for ELISA and cells were lysed for RNA isolation.

In some experiments the keratinocytes were stimulated for 20 h with culture supernatants of *S. epidermidis*. These supernatants were generated by incubating 8 mL (approx. 5×10^6 CFU/mL) of *S. epidermidis* in KGM2 in a petri dish at 37 °C for 24 h. Subsequently, the bacteria were pelleted by centrifugation at 8.500 *g* and the remaining supernatant was sterile filtered (0.22 μm) and stored at –20 °C until use. For some experiments, size fractionated supernatants were used. To this end, the supernatants were applied to 2- and 3-kDa ultrafiltration centrifugal units (Amicon/Merck, Darmstadt, Germany) and size-fractionated by centrifugation according to the supplier's protocol.

To evaluate the influence of the AhR, the specific AhR inhibitor CH223191 (10 μM, Cayman Chemical Company, Ann Arbor, MI, USA) was used. The inhibitor was applied to the medium 1 h before stimulation and remained during stimulation. DMSO (1:6,000 diluted in medium) served as the vehicle control. To block the activity of NF-κB, we treated the cells with 5 μM of the specific NF-κB inhibitor BMS-345541 (Cayman Chemical Company) and DMSO (1:4,000 diluted in medium) served as the vehicle control.

siRNA Experiments

Primary human keratinocytes were transfected with siRNA (5 nM) at a confluence of 40–60% using 1 μL of the transfection reagent HiPerFect (Qiagen, Hilden, Germany). After 16–20 h the medium was removed and the keratinocytes were further cultured for 3 days until stimulation with *S. epidermidis*. Two different AhR-specific "SilencerSelect" siRNAs (s1199 and s1200) and a nonsilencing control siRNA (4390844) were purchased from Life Technologies (Carlsbad, CA, USA).

Organotypic 3D Skin Equivalent

The organotypic 3D skin equivalent was constructed as recently described [22]. The skin equivalent was stimulated with *S. epidermidis* by application of approximately 2.5×10^7 CFU/mL in 20 μL of KGM2 without supplements for approximately 24 h. During stimulation the culture medium in the external well was treated with DMSO (1:6,000 as vehicle control) or with the specific AhR inhibitor CH223191 (10 μM).

Real-Time PCR Analysis

Total RNA of the keratinocytes was isolated using the reagent Crystal RNAmagic according to the manufacturer's protocol (Bio-

labproducts, Gödenstorf, Germany). In total, 0.5 μg of the isolated RNA was reverse transcribed to cDNA using an oligo dT primer and 12.5 units of reverse transcriptase (PrimeScript RT Reagent Kit, TaKaRa Bio, Saint-Germain-en-Laye, France). cDNA corresponding to 10 ng total RNA served as the template in a real-time PCR. Real-time PCR was performed in a StepOne Real-Time PCR System (Applied Biosystem, Carlsbad, CA, USA) using SYBR Premix Ex Taq II (TaKaRa Bio) as previously described [23]. The following intron-spanning primers were used: IL-1β: 5′-AAG CCC TTG CTG TAG TGG TG-3′ (forward primer) and 5′-GAA GCT GAT GGC CCT AAA CA-3′ (reverse primer); CYP1A1: 5′-CAC CAT CCC CCA CAG CAC-3′ (forward primer) and 5′-ACA AAG ACA CAA CGC CCC TT-3′ (reverse primer); CYP1B1: 5′-TAT CAC TGA CAT CTT CGG CG-3′ (forward primer) and 5′-CTG CAC TCG AGT CTG CAC AT-3′ (reverse primer); IL-1α: 5′-TGT GAC TGC CCA AGA TGA AG-3′ (forward primer) and 5′-AAG TTT GGA TGG GCA ACT GA-3′ (reverse primer); hBD-3: 5′-TGT TTG CTT TGC TCT TCC TGT-3′ (forward primer) and 5′-CGC CTC TGA CTC TGC AAT AA-3′ (reverse primer); AhR: 5′-TCA GTT CTT AGG CTC AGC GTC-3′ (forward primer) and 5′-AGT TAT CCT GGC CTC CGTTT-3′ (reverse primer). Standard curves were generated for each primer set with serial dilutions of cDNA. All quantifications were normalized to the housekeeping gene RPL38 (ribosomal protein L38) using the primer pair: 5′-TCA AGG ACT TCC TGC TCA CA-3′ (forward primer) and 5′-AAA GGT ATC TGC TGC ATC GAA-3′ (reverse primer). Relative expression is given as a ratio between expression of the specific gene and RPL38 gene expression.

AhR and NF-κB Luciferase Gene Reporter Assay

To determine activation of the AhR, we used the *firefly* luciferase reporter plasmid pGUDLUC6.1 (a generous gift from Dr. M. Denison, U.C. Davis). The pGUDLUC6.1 plasmid is derived from the pGUDLUC1.1 plasmid [24] and contains four AhR-responsive elements (dioxin-responsive elements, DREs) and no other known regulatory elements [25]. Human primary keratinocytes were co-transfected using 1 μL of the transfection reagent Fugene HD (Promega, Madison, WI, USA) with 300 ng of pGUDLUC6.1 and 30 ng of a *renilla* luciferase expression plasmid (pGL4.74[hRluc/TK]; Promega), which served as an internal control. After 24 h the cells were stimulated with *S. epidermidis* as described above. Subsequently, keratinocytes were lysed with 150 μL of passive lysis buffer (Promega) and *firefly* and *renilla* luciferase activities were determined using the Dual Luciferase assay system (Promega) on a TD-20/20 luminometer (Promega). Luciferase activity was calculated by the amount of *firefly* luciferase activity normalized to the amount of *renilla* luciferase activity. For determination of NF-κB activity, we performed the same experiment as described above using an NF-κB *firefly* luciferase reporter plasmid instead of pGUDLUC6.1 [23].

ELISA

Secreted protein levels in the cell culture supernatants were measured by specific ELISA for IL-1β and pro-IL-1β (catalog No. DY-201 and DLBP00; R&D Systems, Minneapolis, MN, USA) and for IL-1α (catalog No. 445804; BioLegend, San Diego, CA, USA). ELISA was performed according to the manufacturer's protocol. The detection limit of the IL-1β, pro-IL-1β, and IL-1α ELISA were at 3.4–7.8 pg/mL, 11.6–22.2 pg/mL, and 3.4–7.8 pg/mL, respectively.

Immunostaining

For immunostaining, the organotypic 3D skin equivalent was fixed by formalin and embedded in paraffin. Staining was performed with rabbit anti-IL-1β antibody (1:200, Bioss, Woburn, MA, USA) followed by biotinylated pig anti-rabbit IgG (1:300; Dako Cytomation, Glostrup, Denmark) and avidin/biotinylated enzyme complex (Elite ABC complex, Vector Laboratories, Peterborough, UK) and a horseradish peroxidase substrate (HRP; NovaRed, Vector laboratorie, Burlingame, CA, USA). Slides were counterstained with hematoxylin and mounted with Eukitt (poly[butyl methacrylate-co-methyl methacrylate]; O. Kindler, Freiburg, Germany).

iGLuc Reporter System to Measure Proteolytic Cleavage of Pro-IL-1β

To analyze whether the AhR may influence proteolytic processing of pro-IL-1β, we transfected the keratinocytes with a luciferase-based inflammasome and protease activity reporter termed iGLuc [26]. This reporter, which contains *Gaussia* luciferase fused to a part of pro-IL-1β, releases luciferase activity in the supernatant of the keratinocytes upon proteolytic cleavage of pro-IL-1β. This plasmid (300 ng/well) was transfected in the keratinocytes 24 h before stimulation using 1 μL of Fugene HD (Promega). After stimulation, the cell culture supernatant was harvested and luciferase activity was determined on a TD-20/20 luminometer (Promega) by mixing a 4.4-μM coelenterazine (Carl Roth, Karlsruhe, Germany) solution with the cell culture supernatant at the ratio of 1:1 (35 μL).

Ex vivo Skin Explant Model for Studying the Influence of the AhR on S. epidermidis *Growth*

Human skin explants were derived from abdomen or breast reduction surgeries after written informed consent was obtained. The use of skin material for this study was approved by the local ethics committee of the Medical Faculty, University of Kiel, Germany (D 414/09; D 442/16). Skin samples were washed with PBS, subcutaneous fat was carefully removed and the samples were cut in defined pieces (0.25 cm^2). For stimulation a 1.5-mL microtube was filled with 400 μL of KGM2 without supplements and the specific AhR inhibitor CH223191 (10 μM) or DMSO as vehicle. Subsequently, the skin samples were placed on the medium with the epidermis facing up. Only the dermis was submerged by medium, allowing the epidermis to be exposed to an air interface similar to the in vivo situation. *S. epidermidis* was prepared for stimulation as described above and 20 μL of the bacteria solution (OD_{600} approx. 0.2) was added centrally onto the epidermis. After 5 h of incubation at 37 °C in a 5% CO_2 atmosphere, 600 μL of PBS was added, mixed by vortexing, and the skin was lysed by sonification on ice. Several dilutions were prepared in PBS and 50 μL were plated on tryptic soy broth agar plates. After overnight incubation at 37 °C, the CFUs were counted.

Results

S. epidermidis *Activates an AhR Reporter in Human Primary Keratinocytes*

To determine whether *S. epidermidis* is able to activate the AhR in keratinocytes, we transfected keratinocytes

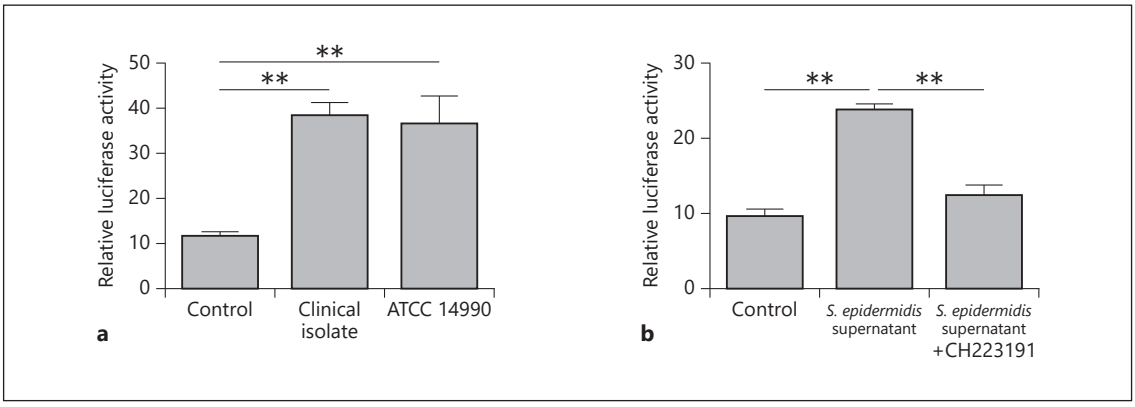

Fig. 1. *S. epidermidis* activates an AhR reporter in human keratinocytes. To investigate whether *S. epidermidis* is able to activate the AhR, we transfected human primary keratinocytes with an AhR *firefly* luciferase reporter plasmid and a *renilla* luciferase control plasmid. The keratinocytes were stimulated with living *S. epidermidis* (clinical isolate) compared to the *S. epidermidis* ATCC strain 14990 (**a**) and with culture supernatants of *S. epidermidis* (clinical isolate) in the presence or absence of the specific AhR inhibitor CH223191 (**b**). AhR activation was determined by analyzing luciferase activity which was determined as the ratio between *firefly* and *renilla* luciferase activities in each sample. Data are the means ± SEM of 3 stimulations (** $p < 0.01$, Student *t* test).

with an AhR luciferase reporter plasmid. Stimulation of these cells with *S. epidermidis* activated the AhR as measured by induction of luciferase activity (Fig. 1a). A clinical isolate as well as an ATCC strain of *S. epidermidis* activated the AhR reporter, suggesting that S. *epidermidis* in general has the capacity to activate the AhR (Fig. 1a). Culture supernatants of *S. epidermidis* also induced the AhR reporter in keratinocytes, indicating that *S. epidermidis* secretes factor(s) activating the AhR. This activation was inhibited by the specific AhR inhibitor CH223191 (Fig. 1b).

S. epidermidis *Activates the AhR-Responsive Genes CYP1A1 and CYP1B1 in Human Primary Keratinocytes*

Stimulation of keratinocytes with *S. epidermidis* induced gene expression of the AhR-responsive gene *CYP1A1* (Fig. 2a). The *S. epidermidis*-mediated gene induction of *CYP1A1* occurred already after 3 h (Fig. 2b). Similarly, another AhR-responsive gene, *CYP1B1*, was also induced by *S. epidermidis* already after 3 h (Fig. 2c). The use of the AhR inhibitor CH223191 completely abolished the *S. epidermidis*-mediated *CYP1A1* induction, confirming the dependency on the AhR (Fig. 2a). Accordingly, *S. epidermidis*-mediated induction of *CYP1A1* gene expression was almost completely blocked in keratinocytes treated with an AhR-specific siRNA (Fig. 2d, e). The use of the *S. epidermidis* strain ATCC 14990 and another AhR-specific siRNA revealed similar results (see online suppl. Fig. 1a, b; for all online suppl. material, see www.karger.com/doi/10.1159/000492162).

Supernatants of three different skin-derived *S. epidermidis* isolates induced CYP1A1 expression in primary keratinocytes, indicating that *S. epidermidis* in general has the capacity to activate AhR signaling in human keratinocytes (see online suppl. Fig. 2a). In addition, supernatants of *S. warneri* and *Corynebacterium amycolatum* induced CYP1A1 gene expression in primary keratinocytes, demonstrating that AhR activation is not restricted to *S. epidermidis* (see online suppl. Fig. 2b).

S. epidermidis *Induces AhR-Dependent IL-1β Expression in Human Primary Keratinocytes*

Stimulation of keratinocytes with *S. epidermidis* resulted in an increased induction of IL-1β gene expression and protein secretion. This induction was significantly inhibited by cotreatment with the specific AhR inhibitor CH223191 (Fig. 3a, b). Similarly, the induction of IL-1β gene expression and protein secretion were significantly inhibited after siRNA-mediated AhR knockdown indicating the participation of the AhR in the *S. epidermidis*-induced IL-1β expression (Fig. 3c, d, and 2d for knockdown efficiency). Similar results were obtained with another AhR-specific siRNA and *S. epidermidis* strain ATCC 14990 (see online suppl. Fig. 3a, b, and online suppl. Fig. 1a for knockdown efficiency). Since the IL-1β ELISA detects 10% of unprocessed pro-IL-1β, we also used an ELISA specific for pro-IL-1β. This revealed that

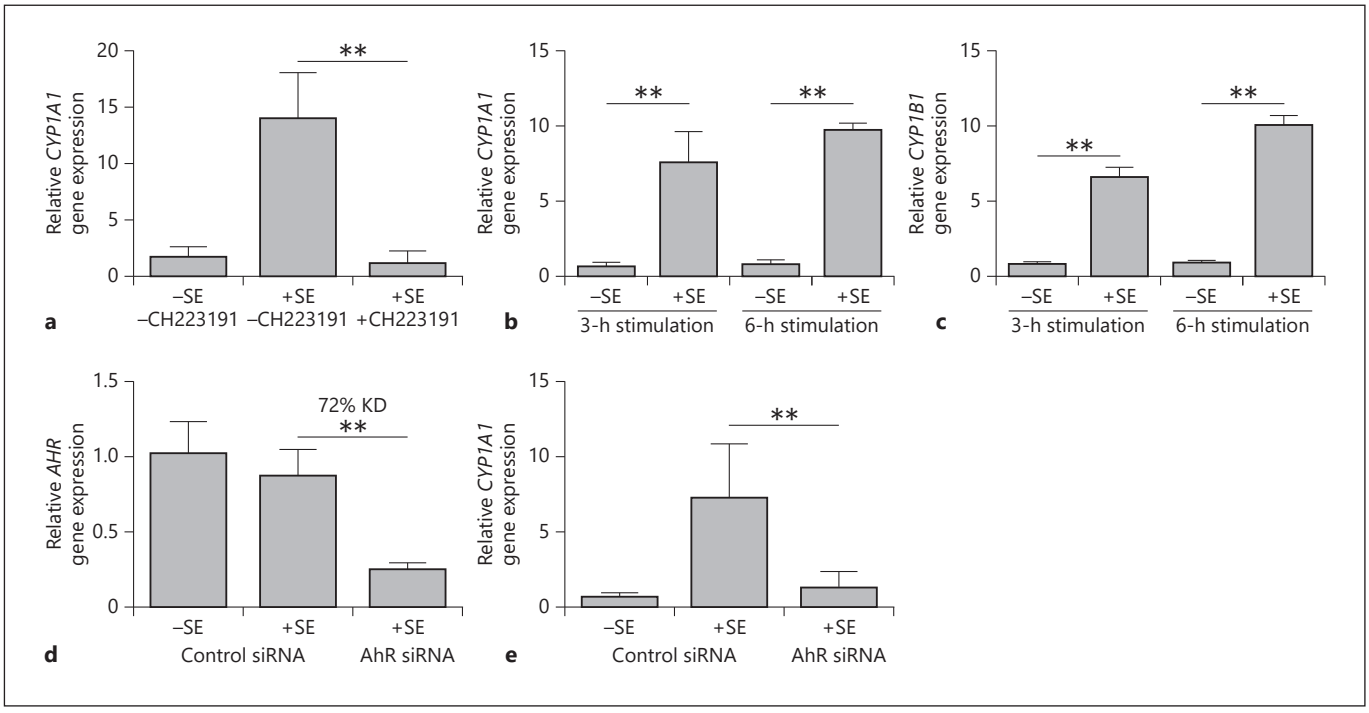

Fig. 2. *S. epidermidis* (SE) induces the AhR-responsive gene CYP1A1 in human keratinocytes. **a** Human primary keratinocytes were stimulated with a clinical isolate of SE in the presence or absence of the specific AhR inhibitor CH223191, and gene expression of CYP1A1 was determined by real-time PCR. **b**, **c** Human primary keratinocytes were stimulated for the indicated time periods with a clinical isolate of SE. Gene expression of *CYP1A1* (**b**) and *CYP1B1* (**c**) were determined by real-time PCR. **d**, **e** Human primary keratinocytes were transfected with a control siRNA or an AhR-specific siRNA (s1199) and stimulated with a clinical isolate of SE. Knockdown efficiency (KD) of AhR gene expression (**d**) and CYP1A1 gene expression (**e**) were analyzed by real-time PCR. Data are the means ± SEM of 6 (**a**, **d**, **e**) or 3 (**b**, **c**) stimulations (** $p < 0.01$, Student *t* test).

the majority of released IL-1β in keratinocytes stimulated with *S. epidermidis* represents the processed mature IL-1β form (see online suppl. Fig. 4).

Activation of the AhR by S. epidermidis *Does Not Influence Processing of IL-1β or NF-κB Activity*

To assess whether the AhR may directly influence proteolytic cleavage of pro-IL-1β, we transfected primary keratinocytes with a luciferase-based inflammasome and protease activity reporter plasmid (iGLuc). Treatment of these keratinocytes with living *S. epidermidis* induced luciferase activation, indicating proteolytic cleavage of IL-1β induced by *S. epidermidis* (see online suppl. Fig. 5a). This activation was not significantly influenced when the cells were cotreated with the specific AhR inhibitor CH223191 (see online suppl. Fig. 5a) although IL-1β secretion was significantly decreased by CH223191 (see online suppl. Fig. 5b). In addition, we also treated the keratinocytes with an AhR-specific siRNA, which resulted in 69% knockdown of AhR gene expression (see online suppl. Fig. 6a). Stimulation of these cells with living *S. epidermidis* induced less IL-1β release, whereas proteolytic cleavage of IL-1β monitored by the iGLuc reporter was not significantly affected by AhR-knockdown (see online suppl. Fig. 6b,c).

To investigate if the *S. epidermidis*-induced activation of the AhR influences NF-κB activity, we transfected human primary keratinocytes with an NF-κB luciferase reporter plasmid. Stimulation of these cells with living *S. epidermidis* induced activation of NF-κB. This activation was not influenced when the cells were cotreated with the specific AhR inhibitor CH223191 (see online suppl. Fig. 7). Since *S. epidermidis* induced the activation of NF-κB, we investigated whether NF-κB was involved in the *S. epidermidis*-mediated IL-1β induction. Blocking NF-κB diminished the induction of IL-1β gene expression in keratinocytes exposed to *S. epidermidis* (see online suppl. Fig. S8).

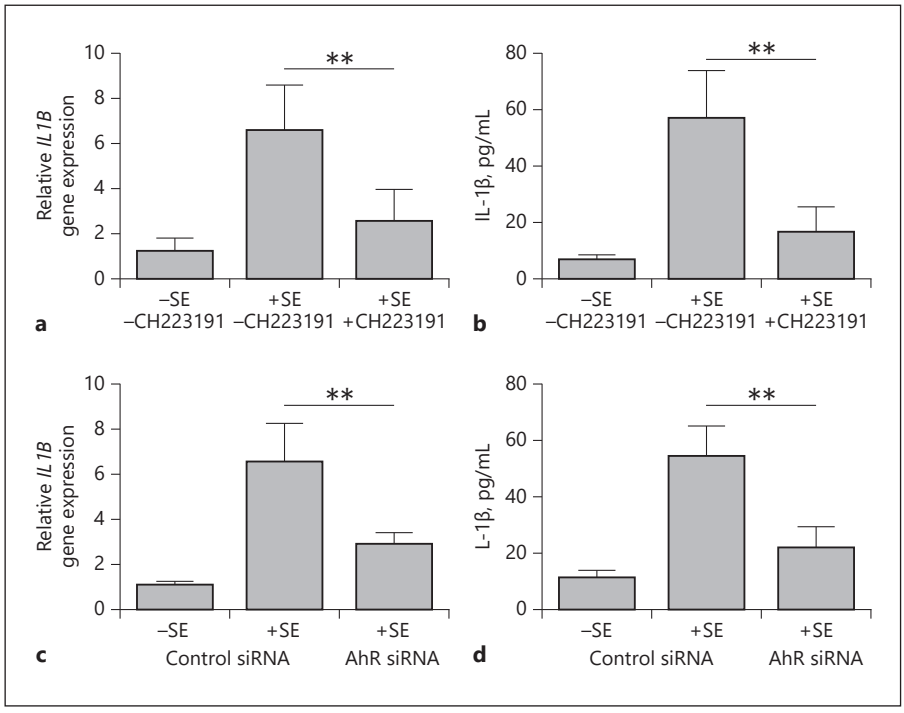

Fig. 3. The AhR mediates *S. epidermidis* (SE)-induced IL-1β expression in human primary keratinocytes. **a**, **b** Human primary keratinocytes were stimulated with a clinical isolate of SE in the presence or absence of the specific AhR inhibitor CH223191. IL-1β gene expression and protein secretion were determined by real-time PCR (**a**) and ELISA (**b**), respectively. **c**, **d** Human primary keratinocytes were transfected with a control siRNA and an AhR-specific siRNA (s1199) and stimulated with a clinical isolate of SE. IL-1β gene expression and protein secretion was determined by real-time PCR (**c**) and ELISA (**d**), respectively. Bars are the means ± SEM of 6 stimulations (** $p < 0.01$; Student *t* test).

S. epidermidis *Culture Supernatants Induce AhR-Dependent CYP1A1 and IL-1β Expression*

Keratinocytes were stimulated with culture supernatants of *S. epidermidis* in the presence of the specific AhR inhibitor CH223191 to explore whether *S. epidermidis* releases factors that induce CYP1A1 and IL-1β in an AhR-dependent manner. This revealed an induction of CYP1A1 gene expression that was blocked by CH223191 (Fig. 4a). Gene expression of CYP1A1 was increased after exposure to <2- and <3-kDa ultrafiltrates of *S. epidermidis* culture supernatants. This induction of CYP1A1 gene expression by the ultrafiltrates of *S. epidermidis* culture supernatants was blocked by CH223191 (Fig. 4a). IL-1β gene expression was also induced after exposure to nonfiltered as well as <2- and <3-kDa ultrafiltrates of *S. epidermidis* culture supernatants (Fig. 4b). This induction was blocked by the specific AhR inhibitor CH223191, indicating the involvement of the AhR in the induction of IL-1β gene expression by *S. epidermidis* culture supernatants. IL-1β protein release by the nonfiltered *S. epidermidis* culture supernatant was also increased and blocked by CH223191 (Fig. 4c). In contrast, the <2- and <3-kDa ultrafiltrates of *S. epidermidis* culture supernatants did not induce release of IL-1β (Fig. 4c).

S. epidermidis *Induces AhR-Dependent CYP1A1 and IL-1β Expression in an Organotypic Skin Equivalent*

Stimulation of an organotypic 3D skin equivalent with *S. epidermidis* in the presence of CH223191 revealed an AhR-dependent induction of CYP1A1 and IL-1β (Fig. 5a–c). In concordance, IL-1β immunostaining of the 3D skin equivalent exposed to *S. epidermidis* showed an intensified IL-1β immunoreactivity, which was reduced by co-treatment with CH223191 (Fig. 5d).

We also investigated whether other defense molecules might be induced by *S. epidermidis* in the 3D skin equivalent in an AhR-dependent manner. We found that the *S. epidermidis*-mediated induction of IL-1α (Fig. 5e, f) and of the antimicrobial peptide hBD-3 (Fig. 5g) were partially blocked after inhibition of the AhR.

The AhR Contributes to Controlling the Growth of S. epidermidis *on Human Skin*

To assess the functional role of the AhR on the interaction between *S. epidermidis* and skin, we investigated if inactivation of the AhR may influence the outgrowth of *S. epidermidis* on the skin surface of human skin explants. To this end, we used an ex vivo model where we applied living *S. epidermidis* for 5 h on the surface of human skin explants. Inactivation of the AhR by CH223191 led to a significant outgrowth of *S. epidermidis* as compared to

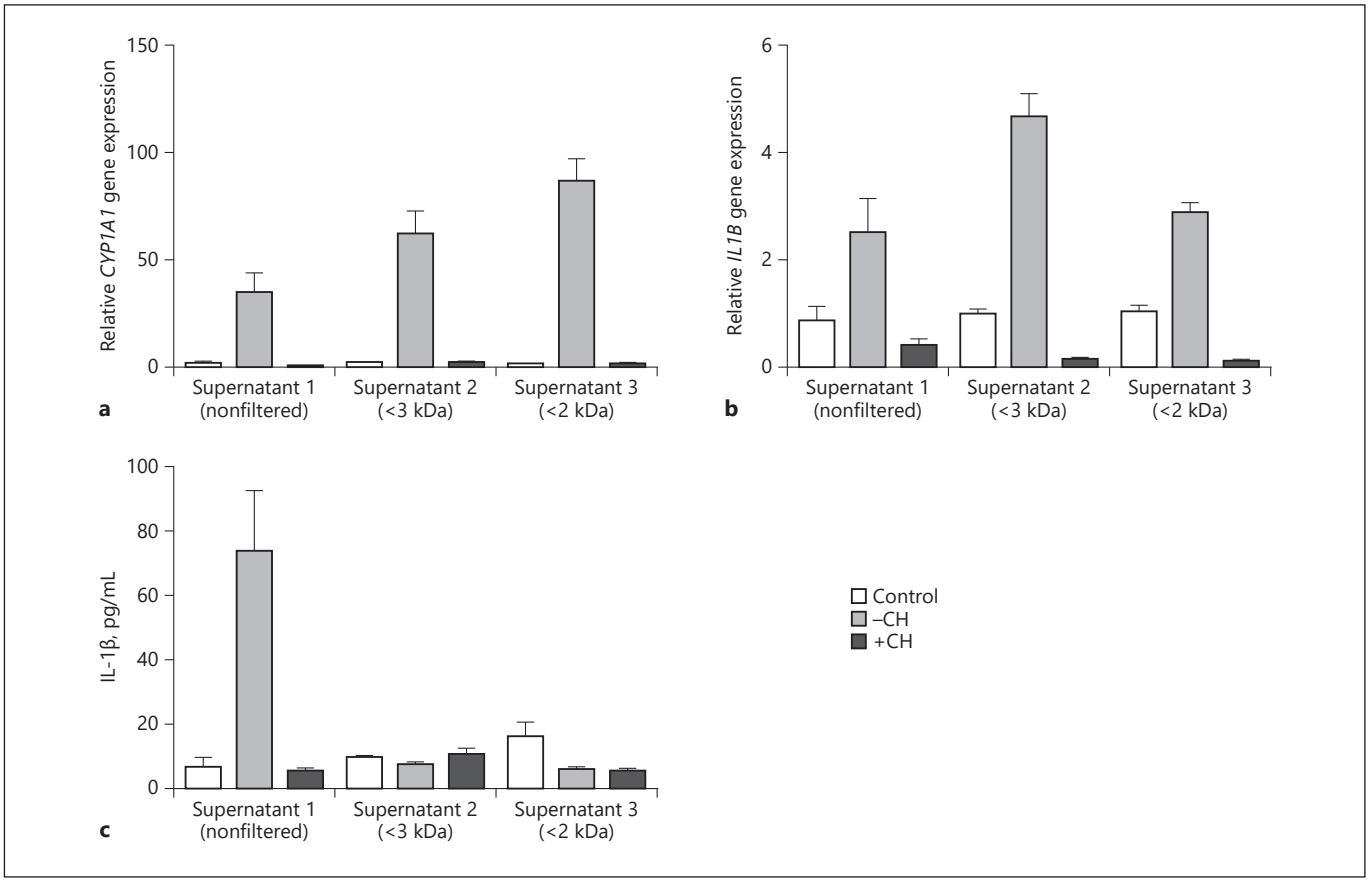

Fig. 4. *S. epidermidis* culture supernatants induce AhR-dependent CYP1A1 and IL-1β expression. Human primary keratinocytes were stimulated without (control) or with different culture supernatants of *S. epidermidis*, either nonfiltered supernatant (supernatant 1) or supernatant with a molecular weight <3 kDa (supernatant 2) or <2 kDa (supernatant 3). Stimulations were done in the absence (–CH) or presence (+CH) of the specific AhR inhibitor CH223191. **a** CYP1A1 gene expression was determined by real-time PCR. **b**, **c** IL-1β gene expression and protein secretion was determined by real-time PCR (**b**) and ELISA (**c**), respectively. Bars are the means ± SEM of 3 stimulations.

skin treated with vehicle only (Fig. 5h). These results indicate a functional relevance of the AhR to controlling the growth of *S. epidermidis* on human skin.

Discussion

S. epidermidis is an abundant member of the human cutaneous microbiota and has been shown to play an important role in skin defense through its activation of cutaneous defense mechanisms [10, 12]. It has been reported that induction of cutaneous defense can be mediated by *S. epidermidis*-induced IL-1 signaling, which promotes protective effector T-cell responses [12]. However, the direct *S. epidermidis*-mediated induction of IL-1 in keratinocytes is less explored. Our results provide evidence that, besides NF-κB, the AhR is crucial for the *S. epidermidis*-mediated gene induction of IL-1β in keratinocytes.

The role of the AhR in skin innate immunity is not well understood. It has been shown that *Malassezia* yeasts are able to secrete various indole derivatives as putative AhR ligands that induced the AhR-responsive genes *CYP1A1* and *CYP1B1* in HaCaT keratinocytes [27]. Nonetheless, it has not been reported whether the AhR plays a role in human keratinocytes in the interaction between keratinocytes and bacteria. Recent research demonstrated that the AhR is able to sense pigmented virulence factors produced by the pulmonary pathogens *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*, thus highlighting a novel role of the AhR as a pattern recognition receptor

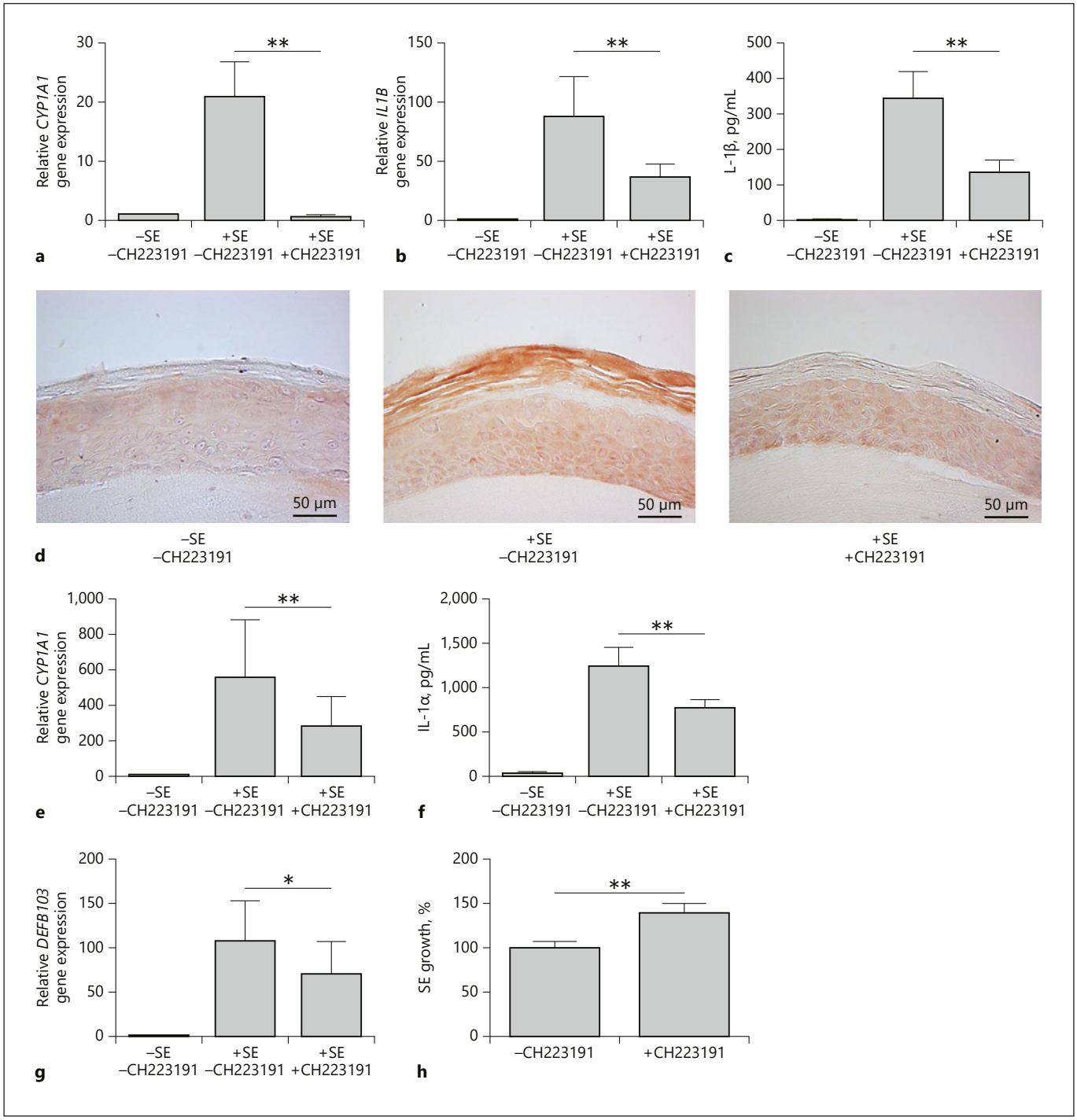

Fig. 5. The AhR mediates activation of skin innate defense by *S. epidermidis* (SE). An organotypic 3D skin equivalent was stimulated with living SE for 24 h in the presence or absence of the AhR inhibitor CH223191 (10 µM). Gene expression of CYP1A1 (**a**) and IL-1β (**b**) was analyzed by real-time PCR. **c** Secretion of IL-1β into the medium was determined by ELISA. **d** Protein expression of IL-1β was analyzed by immunohistochemistry. **e** Gene expression of IL-1α was analyzed by real-time PCR. **f** Secretion of IL-1α into the medium was investigated by ELISA. **g** Gene expression of hBD-3 was analyzed by real-time PCR. Data are the means ± SEM of 7 different skin models (* $p < 0.05$; ** $p < 0.01$; Student ratio paired *t* test). **h** To assess the functional relevance of the AhR to control the growth of SE on the human skin surface, skin explants were exposed to living SE for 5 h in the presence or absence of the specific AhR inhibitor CH223191. The amounts of SE in the presence of the AhR inhibitor CH223191 were compared to the amounts of SE on the skin without treatment, which were set as 100%. Bars are the means ± SEM of 9 skin explants derived from 3 different donors (** $p < 0.01$; Student *t* test).

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[28]. Our data indicate that *S. epidermidis* releases small (<2 kDa) factor(s) that activate the AhR in keratinocytes. Since the known AhR activators are typically derived from small aromatic hydrocarbons, it is likely that *S. epidermidis* releases similar compounds. In addition, it remains to be shown whether the factor(s) released by *S. epidermidis* directly bind to the AhR or increase the amount of endogenous AhR ligands, such as 6-formylindolo[3, 2-b]carbazole (FICZ) through inhibition of CYP450 enzymes [29–31].

There are contradictory studies reporting either a pro- or an anti-inflammatory role of the AhR. Our study demonstrates that antagonizing the AhR leads to a reduced expression of IL-1β in keratinocytes exposed to *S. epidermidis*. In addition, it has been reported that transgenic mice expressing the constitutive active form of the AhR in keratinocytes showed an increased expression of various cytokines, including IL-1β [32]. Iyer et al. [33] reported that AhR activation in intestinal epithelial cells by oxazole compounds induced inflammation. In line with a proinflammatory role of the AhR, the induction of IL-1β in leukocytes derived from bronchoalveolar lavage fluid of *P. aeruginosa*-infected mice was reduced in AhR-deficient cells [28]. On the other hand, other studies reported that AhR-deficient cells showed an exacerbated expression of cytokines in reaction to inflammatory stimuli. For example, mouse macrophages treated with AhR-siRNA expressed more IL-1β mRNA upon LPS stimulation [34]. Moreover, in mouse skin, the lack of AhR caused hyperinflammation, and AhR signaling exerted an anti-inflammatory effect in keratinocytes [19]. These contrasting outcomes of AhR activation may be related to the fact that AhR ligands may serve as activators or suppressors of subsequent signal transduction pathways depending on the nature and concentration of the ligands and the cell-specific environment [13, 35]. In this context, it has been reported that different tryptophan-derived metabolites may act as AhR agonists and antagonists [36]. Furthermore, isoflavones have been identified as AhR agonists and antagonists depending on the species and cell-specific context [37]. The recent demonstration that indole and 3-methyl indole are potent ligands and activators of the human AhR, but only weak activators of the mouse AhR, highlights the importance of also considering potential differences between activation of the AhR in humans and mice [38].

Recently, it has been reported that the inflammasome activation in mouse macrophages by LPS in combination with nigericin, ATP, or alum was inhibited by activation of the AhR through the dioxin-derivative TCDD [34]. Therefore, we sought to determine whether the inflammasome is affected by AhR in our experimental system. To this end, we transfected the keratinocytes with a luciferase-based inflammasome and protease activity reporter (iGLuc). This reporter indicates proteolytic processing of IL-1β [26]. Blocking AhR activity by the use of the specific AhR inhibitor CH223191 as well as by AhR-specific siRNA did not significantly affect *S. epidermidis*-induced iGLuc activity. These data indicate that the AhR has no direct influence on proteolytic processing of pro-IL-1β in the keratinocytes stimulated with *S. epidermidis*.

As *S. epidermidis* is able to activate the transcription factor NF-κB and because a cross-talk of the AhR and NF-κB has been described [39], we investigated whether the *S. epidermidis*-mediated AhR activation may influence NF-κB. Although we observed that AhR activation in keratinocytes by *S. epidermidis* did not directly influence NF-κB activation, we found that activation of both transcription factors, NF-κB and AhR, were crucial for the *S. epidermidis*-mediated induction of IL-1β in keratinocytes. This is in concordance with the necessity of NF-κB and AhR for IL-1β expression in TLR- and *M. tuberculosis*-activated dendritic cells and macrophages [40, 41].

In addition to IL-1β, we also found that other *S. epidermidis*-induced defense molecules are regulated via the AhR. In particular, the *S. epidermidis*-induced expression of IL-1α and the antimicrobial peptide hBD-3 in the 3D organotypic skin depended on the AhR. These data indicate that the AhR mediates the expression of diverse innate defense molecules in keratinocytes, thus highlighting an important role of the AhR in innate cutaneous defense. This is supported by the observed outgrowth of *S. epidermidis* on the surface of skin explants treated with an AhR inhibitor.

In conclusion, our study provides novel insight into the molecular interaction between the skin commensal *S. epidermidis* and keratinocytes, and further strengthens the hypothesis that the AhR evolved not only to sense environmental toxins, but also to participate in host-microbe interactions. Understanding how commensals modulate host defense may provide innovative strategies for the treatment or prophylaxis of infectious and inflammatory diseases.

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Disclosure Statement

The authors declare no conflicts of interest.

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