Staphylococcus aureus Activates the Aryl Hydrocarbon Receptor in Human Keratinocytes

Eva-Lena Stange  Franziska Rademacher  Katharina Antonia Drerup  
Nina Heinemann  Lena Möbus  Regine Gläser  Jürgen Harder

Department of Dermatology, Kiel University, Kiel, Germany

Abstract
Staphylococcus aureus is an important pathogen causing various infections, including – as most frequently isolated bacterium – cutaneous infections. Keratinocytes as the first barrier cells of the skin respond to S. aureus by the release of defense molecules such as cytokines and antimicrobial peptides. Although several pattern recognition receptors expressed in keratinocytes such as Toll-like and NOD-like receptors have been reported to detect the presence of S. aureus, the mechanisms underlying the interplay between S. aureus and keratinocytes are still emerging. Here, we report that S. aureus induced gene expression of CYP1A1 and CYP1B1, responsive genes of the aryl hydrocarbon receptor (AhR). AhR activation by S. aureus was further confirmed by AhR gene reporter assays. AhR activation was mediated by factor(s) <2 kDa secreted by S. aureus. Whole transcriptome analyses and real-time PCR analyses identified IL-24, IL-6, and IL-1beta as cytokines induced in an AhR-dependent manner in S. aureus-treated keratinocytes. AhR inhibition in a 3D organotypic skin equivalent confirmed the crucial role of the AhR in mediating the induction of IL-24, IL-6, and IL-1beta upon stimulation with living S. aureus. Taken together, we further highlight the important role of the AhR in cutaneous innate defense and identified the AhR as a novel receptor mediating the sensing of the important skin pathogen S. aureus in keratinocytes.

Introduction
Staphylococcus aureus is a gram-positive, coagulase-positive bacterium that forms biofilms and causes opportunistic infections in various tissues, including skin [1]. S. aureus is temporarily found on human skin where its presence is associated with a higher risk for subsequent infections [2, 3]. Cutaneous colonization and infection with S. aureus is also a typical hallmark of the chronic inflammatory skin disease atopic dermatitis (AD) and AD skin is more frequently colonized by S. aureus than healthy skin [4].

Eva-Lena Stange and Franziska Rademacher contributed equally to this work.

Keywords
Host defense · Keratinocytes · Aryl hydrocarbon receptor · Staphylococcus aureus
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In a previous study, we found evidence that the skin commensal Staphylococcus epidermidis activates the aryl hydrocarbon receptor (AhR) in keratinocytes [9]. The AhR is a ligand-activated transcription factor involved in xenobiotic metabolism, epidermal barrier formation, immune signaling, and immune cell differentiation [10–12]. AhR is activated upon binding of various low-molecular-weight ligands; the receptor is expressed in various tissues, particularly high expression is found in the liver and in barrier organs such as gut and skin [13, 14]. There is increasing evidence that the AhR plays a major role in host defense [11, 13, 15]. The AhR can be activated by metabolites of bacteria such as Pseudomonas aeruginosa [10] or members of the skin microbiota, such as Malassezia yeasts [16]. Although the role of the AhR in cutaneous defense is still emerging there is growing evidence that it plays an important role in skin-microbe interaction [14]. Several reports have shown that the AhR is crucial for the maintenance of skin barrier function [17, 18]. AhR activation by coal tar or the AhR activator tapinarof has been reported to ameliorate AD symptoms by restoring the skin barrier [18, 19]. In addition, activation of the AhR by microbial tryptophan metabolites has been associated with attenuation of inflammation in AD patients [20]. On the other hand, it has been reported that AhR expression in AD skin correlated with the severity of AD symptoms [21].

In this study, we provide evidence that the AhR in keratinocytes is activated by S. aureus and that the expression of several inflammatory cytokines induced by S. aureus is mediated by the AhR. This strengthens the role of the AhR as an innate microbial sensor and a mediator of the innate immune defense of human skin.

Materials and Methods

Keratinocyte Cell Culture and Stimulation

Normal human primary keratinocytes (NHEKs), pooled from four donors (Promocell, Heidelberg, Germany) were cultured in Keratinocyte Growth Medium 2 (KGM2; Promocell), including supplements and CaCl₂ at 37°C/5% CO₂ in 24-well plates until post-confluence. S. aureus skin-derived clinical isolates (identity verified by MALDI-TOF mass spectrometry; MALDI Biotyper; Bruker, Billerica, MA, USA) and S. aureus ATCC 8525-4 were grown on blood agar plates for 24 h and then inoculated into tryptic soy broth and grown under agitation for 16–18 h at 37°C. 250 μL of the bacterial suspension was inoculated into 7 mL tryptic soy broth and further grown for 3–4 h. Bacteria were centrifuged for 5 min at 4,500 g, the pellet was washed with 7 mL phosphate-buff ered saline (PBS) and then the OD600 was adjusted to 0.2 in KGM2 medium (without supplements, with CaCl₂) corresponding to approx. 1.7 x 10⁸ bacteria/mL. This suspension was diluted 1:2 with KGM2 and each well of NHEKs was stimulated with 300 μL. 3 h after the start of the stimulation, the medium was discarded, NHEKs were washed once with PBS and incubated with 300 μL KGM2 supplemented with 200 μg/mL gentamicin sulfate to kill any remaining extracellular bacteria. NHEKs were stimulated for another 14–16 h and then the medium was removed, centrifuged at 12,000 g for 5 min, and stored at −80°C for ELISA analyses. Keratinocytes were also stimulated with S. aureus culture supernatants and size filtered supernatants (prepared as described below). After stimulation with living bacteria or bacterial culture supernatants, keratinocytes were washed with PBS and used for RNA isolation.

In some experiments, the AhR was inhibited by using the AhR inhibitor CH-223191 (Cayman Chemicals). To this end, NHEKs were preincubated with 10 μM CH-223191 for 1–1.5 h before the start of the stimulation and then stimulated in the presence of 10 μM CH-223191. 0.1% DMSO served as vehicle control.

Production of Bacterial Culture Supernatants

S. aureus was adjusted to an OD 600 nm of 0.2 in KGM2 medium as described above. Eight milliliters of this suspension was filled into sterile petri dishes and incubated for 24 h at 37°C. Subsequently, the bacteria suspension was harvested and centrifuged for 5 min at 8,500 g. The supernatant was sterile filtered (0.2 μm pore size) and stored at −20°C until use in stimulation experiments. For size filtration, the supernatant was applied to 2 kDa centrifugal concentrators (Vivaspin 15 R Hydrosart filter device; Sartorius, Göttingen, Germany) and centrifuged for 1 h at 3,000 g according to the suppliers’ protocol. The >2 kDa concentrate was washed three times with KGM2. Filtrate and concentrate were used for stimulation of NHEKs diluted 1:2 in KGM2.

AhR Gene Reporter Luciferase Assay

To test nuclear translocation and binding of the AhR to AhR-responsive elements, the firefly luciferase reporter plasmid pGUD-LUC6.1 (generously gifted by M. Denison, U.C. Davis) was used. This plasmid contains 4 AhR-responsive elements and no other known regulatory elements [22]. 300 ng of this plasmid together with 30 ng of a renilla luciferase control plasmid (pGL4.74[hRluc/TK]; Promega, Madison, WI, USA) were transfected in keratinocytes (24 wells, cultured with 400 μL KGM2) using the transfection reagent Fugene HD (Promega). 24 h after transfection, cells were stimulated with S. aureus as described above. After stimulation, cells were lysed with passive lysis buffer (Promega), and firefly and renilla luciferase activities were determined using the Dual Luciferase assay system (Promega). Specific AhR-luciferase activity was determined by normalizing the firefly luciferase activity to renilla luciferase activity.

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**Fig. 1.** *S. aureus* induces AhR-luciferase reporter activity. NHEKs were transfected with an AhR *firefly* luciferase reporter plasmid (pGudLuc6.1) and a *renilla* luciferase control plasmid (hRLuc/TK). Two days later the cells were stimulated with living *S. aureus* (clinical isolates SA 129 and SA 178, ATCC strain 8325-4 and 6.25 μM pyocyanin as positive control). AhR activation was determined by measuring luciferase activity, which was calculated as the ratio of *firefly* and *renilla* luciferase activities. Shown are means ± SEM (n = 12–18 stimulations, ***p < 0.001, Mann-Whitney U test).

**AhR siRNA Experiments**

NHEKs were transfected at 50%–70% confluency with 1 μL HiPerfect transfection reagent (Qiagen) and 5 nM of either AhR-specific “SilencerSelect” siRNA (s1199) or nonsilencing control siRNA (4390844) purchased from Life Technologies (Carlsbad, CA, USA). After 24 h of incubation with the siRNA, medium was changed and cells were grown for 3 additional days until stimulation.

**3D Organotypic Skin Equivalent**

The organotypic 3D skin equivalent was constructed as previously described [23]. The skin equivalent was preincubated with 10 μM CH-223191 or the corresponding volume of DMSO as a solvent control for 1–1.5 h. Stimulation with *S. aureus* SA 129 was done by application of approximately 1.2 × 10^8 CFU/mL in 20 μL of KGM2 without supplements onto the skin equivalent. Stimulation was done for approximately 24 h at 37°C/5% CO₂.

**Real-Time PCR Analysis**

Total RNA of the keratinocytes was isolated using the reagent Crystal RNAmagic according to the manufacturer’s protocol (Biola-products, Hamburg, Germany). 0.5 μg of the isolated RNA was reverse transcribed to cDNA using an oligo dT primer and 12.5 units of reverse transcriptase mix (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 with the QuantStudio 3 System (BD Biosciences) using SYBR Premix Ex Taq II mix (TaKaRa Bio, Saint-Germain-en-Laye, France). cDNA was reverse transcribed to cDNA using an oligo dT primer and 12.5 units of reverse transcriptase mix (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.

**Whole Transcriptome Sequencing**

In this pilot study, human primary keratinocytes were stimulated with *S. aureus* clinical isolate SA 179 for 20 h in the presence or absence of the AhR inhibitor CH-223191 (n = 5 of each treatment). Total RNA was isolated with the NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. RNA libraries were prepared and sequenced on a HiSeq4000 (Illumina, San Diego, CA, USA) and analyzed as described recently [24].

**Immunostaining**

The organotypic 3D skin equivalent was fixed by formalin and embedded in paraffin. For immunostaining goat anti-IL-24 antibody (R&D Systems, Minneapolis, MN, USA) 1:100 diluted in TBS/1% BSA was used as primary antibody. Biotinylated rabbit anti-goat (Jackson ImmunoResearch, UK) 1:500 diluted in TBS/1% BSA served as second antibody. Blocking was done with TBS/1% BSA. For development, the Vectastain Elite ABC Komplex (PK-6100) followed by the Vector Nova Red-Substrate (SK-4800; both Vector Laboratories, USA) was used. Slides were counterstained with hematoxylin and mounted with Eukitt (Poly(butyl methacrylate-co-methyl methacrylate); O. Kindler, Freiburg, Germany).

**Statistics**

Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). D’Agostino & Pearson test was used to analyze the distribution of the data. Normally distributed data were analyzed by t test (comparison of two groups) or ANOVA with Sidak’s multiple comparisons test. Otherwise, a nonparametric Mann-Whitney test (comparison of two groups) or Kruskal-Wallis test with Dunn’s multiple comparisons test was used. A p value <0.05 was considered statistically significant.
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(For legend see next page.)
Results

S. aureus Bacteria Induce AhR-Luciferase Reporter Activity

To analyze if S. aureus can activate the AhR, we transfected NHEKs with an AhR-luciferase reporter plasmid and stimulated the cells with different S. aureus strains: the clinical isolate SA 129 from the skin of a healthy person, the clinical isolate SA 178 from lesional skin of an AD patient and the ATCC reference strain 8325-4. All strains increased AhR reporter luciferase activity in comparison to unstimulated NHEKs (shown in Fig. 1). For strain, SA 129 and ATCC 8325-4 this increase was similar to reporter luciferase activity in NHEKs stimulated with the AhR activator pyocyanin [10] which was used as a positive control in this experiment.

S. aureus Induces AhR Target Gene Expression in Primary Keratinocytes

Stimulation of NHEKs with the clinical isolates SA 129 and SA 178 induced the AhR-responsive genes CYPIA1 and CYP1B1. This induction was completely abrogated in the presence of the AhR inhibitor CH-223191 (shown in Fig. 2). To gain further insight into the potential influence of the AhR in S. aureus-induced genes we performed a whole transcriptome analysis of NHEKs stimulated with S. aureus clinical isolate SA 178 in the presence or absence of the AhR inhibitor CH-223191. This approach identified several S. aureus-induced genes whose induction was inhibited by blocking the AhR through CH-223191 (shown in online suppl. Table 1; see www.karger.com/doi/10.1159/000524033 for all online suppl. material). Based on this analysis, we have chosen the cytokines IL-24 and IL-6 for further verification by real-time PCR because the transcriptome sequencing revealed a high IL-24 and IL-6-induced expression of S. aureus (shown in Fig. 3). To this end, we transfected NHEKs with an AhR-luciferase reporter plasmid and stimulated the cells with culture supernatants of S. aureus SA 178. This revealed an enhanced luciferase activity indicating activation of the AhR (shown in Fig. 4).

S. aureus Culture Supernatants Induce AhR Target Gene Expression in Primary Keratinocytes

We next sought to determine whether the observed AhR-dependent S. aureus-mediated induction of AhR target genes was mediated by factor(s) released by S. aureus. To this end, we transfected NHEKs with an AhR-luciferase reporter plasmid and stimulated the cells with culture supernatants of S. aureus isolates SA 129 and SA 178. This revealed an enhanced luciferase activity indicating activation of the AhR (shown in Fig. 4).

Subsequently, we stimulated NHEKs with culture supernatants of S. aureus SA 129 and SA 178 for 6 h and 17 h and analyzed gene expression of the AhR-responsive genes CYPIA1 and CYP1B1 by real-time PCR. Induction was seen only after 17 h (shown in Fig. 5a, b). Stimulation of the NHEKs with <2 and >2 kDa ultrafiltrates of S. aureus culture supernatants revealed induction of CYPIA1 only with the >2 kDa ultrafiltrate. This induction was blocked by CH-223191 (shown in Fig. 5c). These data indicate that the AhR-inducing activity is present in the <2 kDa ultrafiltrate.
**Fig. 3.** *S. aureus* induces AhR target gene expression in 3D skin equivalents. **a** 3D skin equivalents were stimulated for 20–24 h with living *S. aureus* clinical isolate SA 129 in the presence or absence of the AhR inhibitor CH223191 (CH). Gene expression of the AhR-responsive genes CYP1A1 and CYP1B1 as well as the cytokines IL-24, IL-6, and IL-1beta was analyzed by real-time PCR. IL-1beta protein secretion was measured by ELISA. Shown are cumulative data of 5 skin equivalents (means + SEM; *p* < 0.05, **p** < 0.01). **b** Protein expression of IL-24 was analyzed by immunohistochemistry.
We next inhibited the expression of the AhR in NHEKs by transfection of the cells with an AhR-specific siRNA. This revealed a knockdown of AhR expression of 85% (shown in Fig. 5d). Stimulation of the AhR-siRNA-treated NHEKs with culture supernatant of \textit{S. aureus} SA 178 revealed decreased induction of IL-24, IL-6, and L-1beta (shown in Fig. 5d). These data show that \textit{S. aureus} secretes factor(s) that induce the cytokines IL-24, IL-6, and L-1beta in an AhR-dependent manner.

**Discussion**

The role of the AhR in cutaneous defense is still emerging. Recent studies reporting that bacteria such as \textit{P. aeruginosa} [10] and \textit{S. epidermidis} as well as \textit{Malassezia} yeasts [16] activate the AhR strengthen the hypothesis that the AhR may serve as an ancient pattern recognition receptor. Moreover, a recent mouse study has shown that murine skin lacking AhR signaling displayed enhanced epidermal barrier defects. Interestingly, topical colonization with a mix of defined bacterial skin commensals (\textit{S. epidermidis}, \textit{S. hemolyticus}, \textit{S. warneri}, \textit{Micrococcus luteus}, \textit{Corynebacterium aurimucosum}) restored epidermal barrier function. This study highlights an important role of the AhR in the epidermal barrier-microbiota interplay and provides further evidence of a crucial role of the AhR in bacterial sensing [25].

In the present study, we show for the first time that the AhR is involved in the recognition of the important skin pathogen \textit{S. aureus} by keratinocytes. Various \textit{S. aureus} strains were able to induce expression of the AhR-responsive gene CYP1A1 in keratinocytes indicating that \textit{S. aureus} in general has the capacity to activate the AhR. Thus, the AhR may play a major role in the interplay of keratinocytes and \textit{S. aureus} and may act as a pattern recognition receptor to sense the presence of \textit{S. aureus}. Our data show that the AhR-activating factor(s) released by \textit{S. aureus} have a molecular weight <2 kDa which is in line with the characteristics of small aromatic hydrocarbons as AhR ligands. It is known that tryptophan metabolites act as ligands of the AhR [26] and such tryptophan-derived AhR ligands may be produced by \textit{S. aureus}, a hypothesis that remains to be proven. A recent study showed that peptidoglycan, a bacterial ligand of toll-like receptor-2 (TLR-2), led to increased CYP1A1 gene expression in keratinocytes indicating activation of AhR signaling. Given the size of peptidoglycan, it is rather unlikely that it serves as a direct AhR ligand. Accordingly, the authors of that study assume that peptidoglycan may indirectly activate AhR signaling through stimulated production of endogenous AhR ligands [27].

There is increasing evidence that therapeutically targeting the AhR may ameliorate skin-associated inflammatory scenarios as seen in the chronic inflammatory skin diseases psoriasis and AD [28]. On the other hand, AhR expression is induced in psoriasis and AD [29], and mice constitutively overexpressing AhR in keratinocytes reveal a disturbed epidermal barrier and increased inflammation that resembled typical AD [30].

**Fig. 4.** \textit{S. aureus} culture supernatants induce AhR-luciferase reporter activity. NHEKs were transfected with an AhR firefly luciferase reporter plasmid (pGudLuc6.1) and a renilla luciferase control plasmid (hRLuc/TK). 48 h later the cells were stimulated with culture supernatants (1:5 dilution) of \textit{S. aureus} clinical isolates SA 129 and SA 178 for 16–18 h. AhR activation was determined by measuring luciferase activity, which was calculated as the ratio of firefly and renilla luciferase activities. Shown are means + SEM ($n = 13$ [SA 129] and $n = 16$ [SA 178]; * $p < 0.05$, *** $p < 0.001$, Mann-Whitney U test).
Fig. 5. *S. aureus* culture supernatants induce AhR target gene expression. NHEKs were stimulated with culture supernatants of *S. aureus* isolates SA 129 (a) and SA 178 (b) for 6 h and 17 h with or without the AhR inhibitor CH-223191 (CH). c NHEKs were stimulated with culture supernatants of *S. aureus* isolates SA 178, either whole non-filtered supernatant or supernatant with a molecular weight < or >2 kDa. d NHEKs were transfected with a control siRNA and an AhR-specific siRNA and stimulated with culture supernatants of SA 178. Relative gene expression was analyzed by real-time PCR. Bars are means ± SEM of three (a–c) or six (d) stimulations (*p < 0.05, **p < 0.01, ***p < 0.001).
hypothesized that under specific pro-inflammatory conditions AhR signaling might be compromised and thus restoration of AhR signaling by AhR agonist may offer a beneficial treatment strategy. In other conditions where an environmental over-activation of the AhR takes place, it would be preferable to dampen AhR signaling. This may also play a role in the prevention of skin cancer and skin aging [31].

We have shown that *S. aureus* induces IL-24 in keratinocytes, a process that required activation of the AhR. This implies that activation of the AhR in general may lead to increased IL-24 levels. In line with these data, AhR agonists increased IL-24 in an AhR-dependent manner in lung cells and thus IL-24 may contribute to the inflammatory effects of environmental AhR agonists [32]. Moreover, the AhR agonist tapinarof induced the secretion of IL-24 in keratinocytes and IL-24 negatively regulated expression of the skin barrier proteins filaggrin and loricrin [33]. Despite these inhibitory effects of tapinarof-induced IL-24 on filaggrin and loricrin, both proteins were surprisingly induced in keratinocytes treated with tapinarof [33]. IL-24 activated also the JAK1-STAT3 and MAPK pathways in keratinocytes and induced the secretion of pro-inflammatory mediators IL-8, PGE2, and MMP-1 [34]. In transgenic mice that overexpressed IL-24 in the skin, abnormal epidermal differentiation and proliferation were observed accompanied by increased chemokine production and macrophage infiltration [35]. Accordingly, it has been suggested that topical tapinarof application may promote IL-24 expression by keratinocytes thus promoting skin inflammation [33]. Another study suggested that cytokines targeting the IL-20 receptors type I and II, including IL-24 promote cutaneous *S. aureus* infection in a mouse model by down-regulating IL-1beta and IL-17A dependent pathways. As mentioned in the introduction, increased susceptibility for cutaneous *S. aureus* colonization is associated with AD [4]. Interestingly, elevated IL-24 levels are present in the lesional skin of AD patients [36]. Moreover, a recent transcriptome study using skin biopsies revealed that AhR gene expression positively correlated with AD disease severity scores [21]. Together, these data suggest that activation of the AhR by AhR agonists may trigger inflammatory processes by increased production of IL-24. Our results imply that activation of the AhR by *S. aureus* may promote *S. aureus*-mediated inflammatory processes by increased AhR-dependent production of IL-24, a process that may be relevant in AD and other skin infections. Similarly, we also found an increased AhR-dependent induction of IL-6 and IL-1beta in *S. au-

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**Statement of Ethics**

The isolation of bacteria derived from human skin was approved by the local Ethical Committee of the Medical Faculty at Kiel University (AZ: A 104/06). All study participants gave their written consent. Primary cells were purchased from Promocell (Heidelberg, Germany). The company followed the strictest European and international ethical standards (https://promocell.com/about-us/compliance), including the Declaration of Helsinki. All donors signed an informed consent form.

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.
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Author Contributions

Eva-Lena Stange, Franziska Rademacher, Regine Gläser, and Jürgen Harder conceived and designed the experiments. Eva-Lena Stange, Franziska Rademacher, Katharina Antonia Drerup, Nina Heinemann, and Lena Möbus performed the experiments and acquired the data. Eva-Lena Stange, Franziska Rademacher, Lena Möbus, Regine Gläser, and Jürgen Harder analyzed the data and prepared the figures. Eva-Lena Stange, Franziska Rademacher, Regine Gläser, and Jürgen Harder wrote the paper. All authors discussed the results and commented on the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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