

## ***Research Article***

# ***Staphylococcus aureus activates the Aryl Hydrocarbon Receptor in Human Keratinocytes***

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Short Title: *S. aureus* activates the AhR

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## 1 **Abstract**

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

17

## 18 **Introduction**

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

25 Sensing of *S. aureus* by keratinocytes is the prerequisite to initiate a rapid defense response by the  
26 release of innate defense factors such as antimicrobial peptides (AMP) and cytokines [5, 6]. Although  
27 several pattern recognition receptors such as Toll-like receptor TLR-2 and NOD-like receptor NOD2  
28 have been implicated in the recognition of *S. aureus* by keratinocytes [7, 8], the detailed mechanisms  
29 underlying the sensing of *S. aureus* by keratinocytes are still emerging.

30 In a previous study we found evidence that the skin commensal *Staphylococcus epidermidis* activates  
31 the aryl hydrocarbon receptor (AhR) in keratinocytes [9]. The AhR is a ligand-activated transcription  
32 factor involved in xenobiotic metabolism, epidermal barrier formation, immune signaling and immune  
33 cell differentiation [10-12]. AhR is activated upon binding of various low-molecular-weight ligands; the

34 receptor is expressed in various tissues, particularly high expression is found in the liver and in barrier  
35 organs such as gut and skin [13, 14]. There is increasing evidence that the AhR plays a major role in  
36 host defense [12, 13, 15]. The AhR can be activated by metabolites of bacteria such as *Pseudomonas*  
37 *aeruginosa* [10] or members of the skin microbiota, such as *Malassezia* yeasts [16]. Although the role  
38 of the AhR in cutaneous defense is still emerging there is growing evidence that it plays an important  
39 role in skin-microbe interaction [14]. Several reports have shown that the AhR is crucial for the  
40 maintenance of skin barrier function [17, 18]. AhR activation by coal tar or the AhR activator tapinarof  
41 has been reported to ameliorate AD symptoms by restoring the skin barrier [17, 19]. In addition,  
42 activation of the AhR by microbial tryptophan metabolites has been associated with attenuation of  
43 inflammation in AD patients [20]. On the other hand, it has been reported that AhR expression in AD  
44 skin correlated with the severity of AD symptoms [21].  
45 In this study, we provide evidence that the AhR in keratinocytes is activated by *S. aureus* and that gene  
46 expression of several inflammatory cytokines induced by *S. aureus* is mediated by the AhR. This  
47 strengthens the role of the AhR as an innate microbial sensor and a mediator of the innate immune  
48 defense of human skin.

49

## 50 **Materials and Methods**

### 51 *Keratinocyte cell culture and stimulation*

52 Normal human primary keratinocytes (NHEKs), pooled from four donors (Promocell, Germany) were  
53 cultured in Keratinocyte Growth Medium 2 (KGM2; Promocell) including supplements and CaCl<sub>2</sub> at  
54 37°C/ 5% CO<sub>2</sub> in 24-well plates until post-confluency.

55 *S. aureus* skin-derived clinical isolates (identity verified by MALDI-TOF mass spectrometry; MALDI  
56 Biotyper, Bruker, Billerica, MA, USA) and *S. aureus* ATCC 8325-4 were grown on blood agar plates for  
57 24 h and then inoculated into tryptic soy broth (TSB) and grown under agitation for 16-18 h at 37 °C.  
58 250 µL of the bacterial suspension was inoculated into 7 mL TSB and further grown for 3-4 h. Bacteria  
59 were centrifuged for 5 min at 4.500 x g, the pellet was washed with 7 mL phosphate buffered saline  
60 (PBS) and then the OD<sub>600</sub> was adjusted to 0.2 in KGM2 medium (without supplements, with CaCl<sub>2</sub>)  
61 corresponding to approx. 1.7 x 10<sup>7</sup> bacteria/ml. This suspension was diluted 1:2 with KGM2 and each  
62 well of NHEKs was stimulated with 300 µL. 3 h after the start of the stimulation, the medium was  
63 discarded, NHEKs were washed once with PBS and incubated with 300 µl KGM2 supplemented with  
64 200 µg/mL gentamicin sulfate to kill any remaining extracellular bacteria. NHEKs were stimulated for  
65 another 14-16 h and then the medium was removed, centrifuged at 12.000 x g for 5 minutes and  
66 stored at -80°C for ELISA analyses. Keratinocytes were also stimulated with *S. aureus* culture

67 supernatants and size filtrated supernatants (prepared as described below). After stimulation with  
68 living bacteria or bacterial culture supernatants, keratinocytes were washed with PBS and used for  
69 RNA isolation.

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

74

#### 75 *Production of bacterial culture supernatants*

76 *S. aureus* was adjusted to an OD 600nm of 0.2 in KGM2 medium as described above. 8 ml of this  
77 suspension was filled into sterile petri dishes and incubated for 24 h at 37 °C. Subsequently, the  
78 bacteria suspension was harvested and centrifuged for 5 min at 8.500 x g. The supernatant was  
79 sterile filtered (0.2  $\mu$ m pore size) and stored at -20 °C until use in stimulation experiments. For size  
80 filtration, the supernatant was applied to 2 kDa centrifugal concentrators (Vivaspin 15 R Hydrosart  
81 filter device, Sartorius, Germany) and centrifuged for 1 h at 3000 x g according to the suppliers'  
82 protocol. The > 2 kDa concentrate was washed three times with KGM2. Filtrate and concentrate  
83 were used for stimulation of NHEKs diluted 1:2 in KGM2.

84

#### 85 *AhR gene reporter luciferase assay*

86 To test nuclear translocation and binding of the AhR to AhR-responsive elements, the *firefly*  
87 luciferase reporter plasmid pGUDLUC6.1 (generously gifted by M. Denison, U.C. Davis) was used. This  
88 plasmid contains 4 AhR-responsive elements and no other known regulatory elements [22]. 300 ng of  
89 this plasmid together with 30 ng of a *renilla* luciferase control plasmid (pGL4.74[hRluc/TK], Promega)  
90 were transfected in keratinocytes (24 wells, cultured with 400  $\mu$ l KGM2) using the transfection  
91 reagent Fugene HD (Promega, Madison, WI). 24 h after transfection, cells were stimulated with *S.*  
92 *aureus* as described above. After stimulation, cells were lysed with passive lysis buffer (Promega) and  
93 *firefly* and *renilla* luciferase activities were determined using the Dual Luciferase assay system  
94 (Promega). Specific AhR luciferase activity was determined by normalizing the firefly luciferase  
95 activity to *renilla* luciferase activity.

96 *AhR siRNA experiments*

97 NHEKs were transfected at 50-70 % confluency with 1  $\mu$ L HiPerfect transfection reagent (Qiagen) and  
98 5 nM of either AhR-specific “SilencerSelect” siRNA (s1199) or nonsilencing control siRNA (4390844)  
99 purchased from Life Technologies (Carlsbad, CA). After 24 h of incubation with the siRNA, medium  
100 was changed and cells were grown for three additional days until stimulation.

101

102 *3D organotypic skin equivalent*

103 The organotypic 3D skin equivalent was constructed as previously described (Rademacher et al.,  
104 2017). The skin equivalent was preincubated with 10  $\mu$ M CH-223191 or the corresponding volume of  
105 DMSO as a solvent control for 1-1.5 h. Stimulation with *S. aureus* SA 129 was done by application of  
106 approximately  $1.2 \times 10^8$  CFU/mL in 20  $\mu$ L of KGM2 without supplements onto the skin equivalent.  
107 Stimulation was done for approximately 24 h at 37 °C/5% CO<sub>2</sub>.

108

109 *Real-time PCR analysis*

110 Total RNA of the keratinocytes was isolated using the reagent Crystal RNAmagic according to the  
111 manufacturer’s protocol (Biolabproducts, Germany). 0.5  $\mu$ g of the isolated RNA was reverse  
112 transcribed to cDNA using an oligo dT primer and 12.5 units of reverse transcriptase mix (PrimeScript  
113 RT Reagent Kit, TaKaRa Bio, Saint-Germain-en-Laye, France). cDNA corresponding to 10 ng total RNA  
114 served as the template in a real-time PCR. Real-time PCR was performed with the QuantStudio3  
115 System (BD Biosciences) using SYBR Premix Ex Taq II mix (TaKaRa Bio) as described [8]. The following  
116 intron-spanning primers were used: IL-1 $\beta$ : 5'-AAG CCC TTG CTG TAG TGG TG-3' (forward primer) and  
117 5'-GAA GCT GAT GGC CCT AAA CA-3' (reverse primer); CYP1A1: 5'-CAC CAT CCC CCA CAG CAC-3'  
118 (forward primer) and 5'-ACA AAG ACA CAA CGC CCC TT-3' (reverse primer); CYP1B1: 5'-TAT CAC TGA  
119 CAT CTT CGG CG-3' (forward primer) and 5'-CTG CAC TCG AGT CTG CAC AT-3' (reverse primer); IL-24:  
120 5'-GTT CCC CAG AAA CTG TGG GA-3 (forward primer) and 5'-CGAGACGTTCTGCAGAACC-3' (reverse  
121 primer); IL-6: 5'- GGT ACA TCC TCG ACG GCA TCT -3' (forward primer) and 5'-GTG CCT CTT TGC TGC  
122 TTT CAC-3' (reverse primer) . Standard curves were produced for each primer set with serial dilutions  
123 of cDNA. All quantifications were normalized to the housekeeping gene RPL38 (ribosomal protein  
124 L38) using the primer pair: 5'- TCA AGG ACT TCC TGC TCA CA-3' (forward primer) and 5'- AAA GGT  
125 ATC TGC TGC ATC GAA-3' (reverse primer).

126

## 127 Whole transcriptome sequencing

128 Human primary keratinocytes were stimulated with *S. aureus* clinical isolate SA 179 for 20 h in the  
129 presence or absence of the AhR inhibitor CH-223191. Total RNA was isolated with the NucleoSpin  
130 RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA libraries  
131 were prepared and sequenced on a HiSeq4000 (Illumina, San Diego, CA, USA) and analyzed as  
132 described recently [23].

133

## 134 Statistics

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

141

## 142 Results

### 143 *S. aureus* bacteria induce AhR-luciferase reporter activity

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

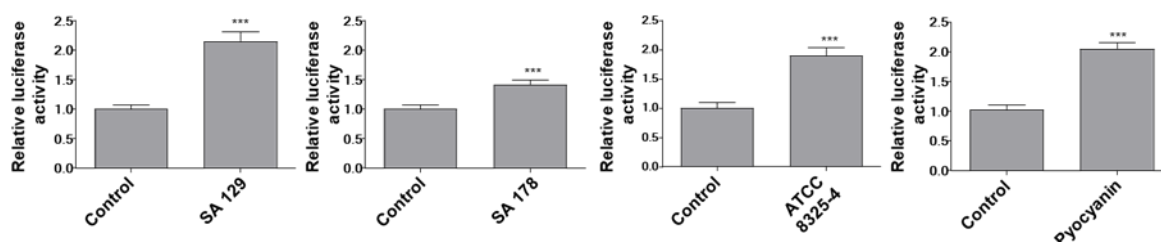
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157 Fig. 1. *S. aureus* induces AhR-luciferase reporter activity. NHEKs were transfected with an AhR *firefly*  
158 luciferase reporter plasmid (pGudLuc6.1) and a *renilla* luciferase control plasmid (hRLuc/TK). Two  
159 days later the cells were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

163

164 *S. aureus* induces AhR target gene expression in primary keratinocytes

165 Stimulation of NHEKs with the clinical isolates SA 129 and SA 178 induced the AhR responsive genes  
166 CYP1A1 and CYP1B1. This induction was completely abrogated in the presence of the AhR inhibitor  
167 CH-223191 (shown in Fig. 2). To gain further insight into the potential influence of the AhR in *S.*  
168 *aureus*-induced genes we performed whole transcriptome analysis of NHEKs stimulated with *S.*  
169 *aureus* clinical isolate SA 178 in the presence or absence of the AhR inhibitor CH-223191. This  
170 approach identified several *S. aureus*-induced genes whose induction was inhibited by blocking the  
171 AhR through CH-223191 (shown in suppl. Table 1). Based on this analysis we have chosen the  
172 cytokines IL-24 and IL-6 for further verification by real-time PCR because the transcriptome  
173 sequencing revealed a high *S. aureus*-induced expression of IL-24 and IL-6, which was inhibited in the  
174 presence of the inhibitor CH-223191. In addition, we analyzed the expression of IL-1beta because our  
175 previous study showed an AhR-dependent induction of IL-1beta in keratinocytes stimulated with *S.*  
176 *epidermidis* [9]. Real-time PCR analyses revealed induction of IL-24, IL-6 and IL-1beta in primary  
177 keratinocytes treated with *S. aureus* isolates SA 129 and SA 178. This induction was inhibited in the  
178 presence of the specific AhR inhibitor CH-223191 (shown in Fig. 2).

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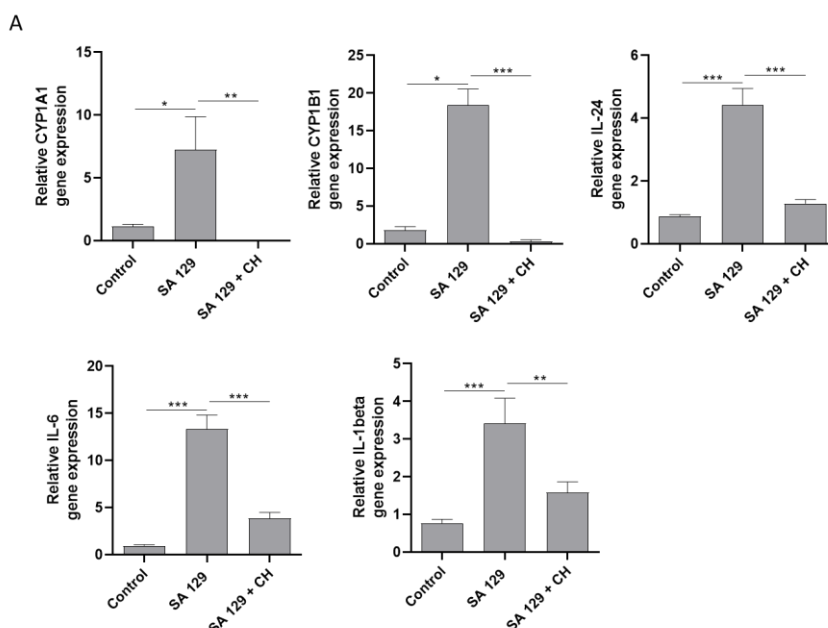
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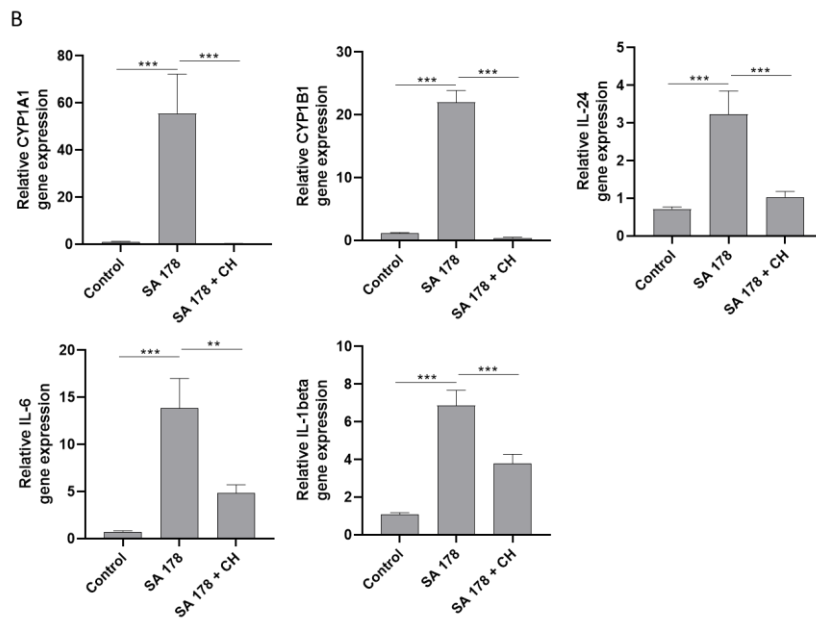
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197 Fig. 2: *S. aureus* clinical isolates induce AhR target gene expression. NHEKs were stimulated with two  
 198 living clinical *S. aureus* isolates SA 129 (a) and SA 178 (b) with or without the AhR inhibitor CH-  
 199 223191. Relative gene expression of the AhR-responsive genes CYP1A1 and CYP1B1 as well as the  
 200 cytokines IL-24, IL-6 and IL-1beta was analyzed by real-time PCR. Shown are cumulative data (means  
 201 + SEM; n=9 (a) and n=15 (b)); \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

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203 To evaluate if activation of the AhR pathway is a general feature of *S. aureus* we screened various *S.*  
 204 *aureus* isolates for their capacity to induce CYP1A1 gene induction in primary keratinocytes. This  
 205 revealed that most strains induced CYP1A1 gene expression (shown in Fig. S1).

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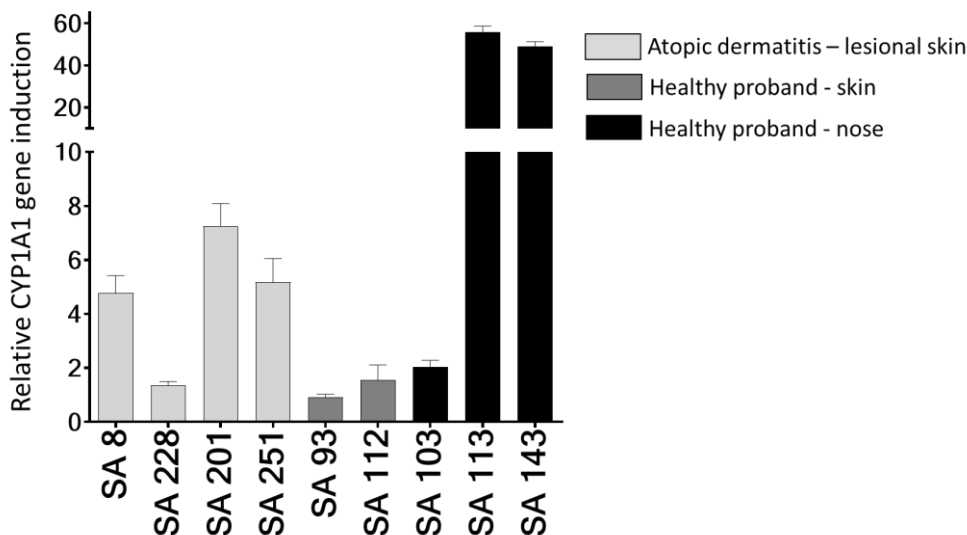
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215 Supplementary Figure 1: Various *S. aureus* clinical isolates induce gene expression of the AhR target  
216 gene CYP1A1. NHEKs were stimulated with different *S. aureus* isolates (SA) derived from lesional skin  
217 of atopic dermatitis patients or derived from the skin or nose from healthy individuals. Stimulation  
218 was done in duplicates and gene expression of CYP1A1 was analyzed by real-time PCR.

219

220 *S. aureus* induces AhR target gene expression in 3D skin equivalents

221 We next stimulated 3D skin equivalents with living *S. aureus* SA 129 in the presence or absence of the  
222 AhR inhibitor CH-223191 and analyzed gene expression by real-time PCR. In line with the results  
223 obtained in the 2D culture, *S. aureus* induced gene expression of the AhR responsive genes CYP1A1  
224 and CYP1B1 as well as the cytokines IL-24, IL-6 and IL-1beta. This induction was inhibited by the AhR  
225 inhibitor CH-223191. IL-1beta protein secretion was also induced by *S. aureus* and inhibited by CH-  
226 223191 (shown in Fig. 3).

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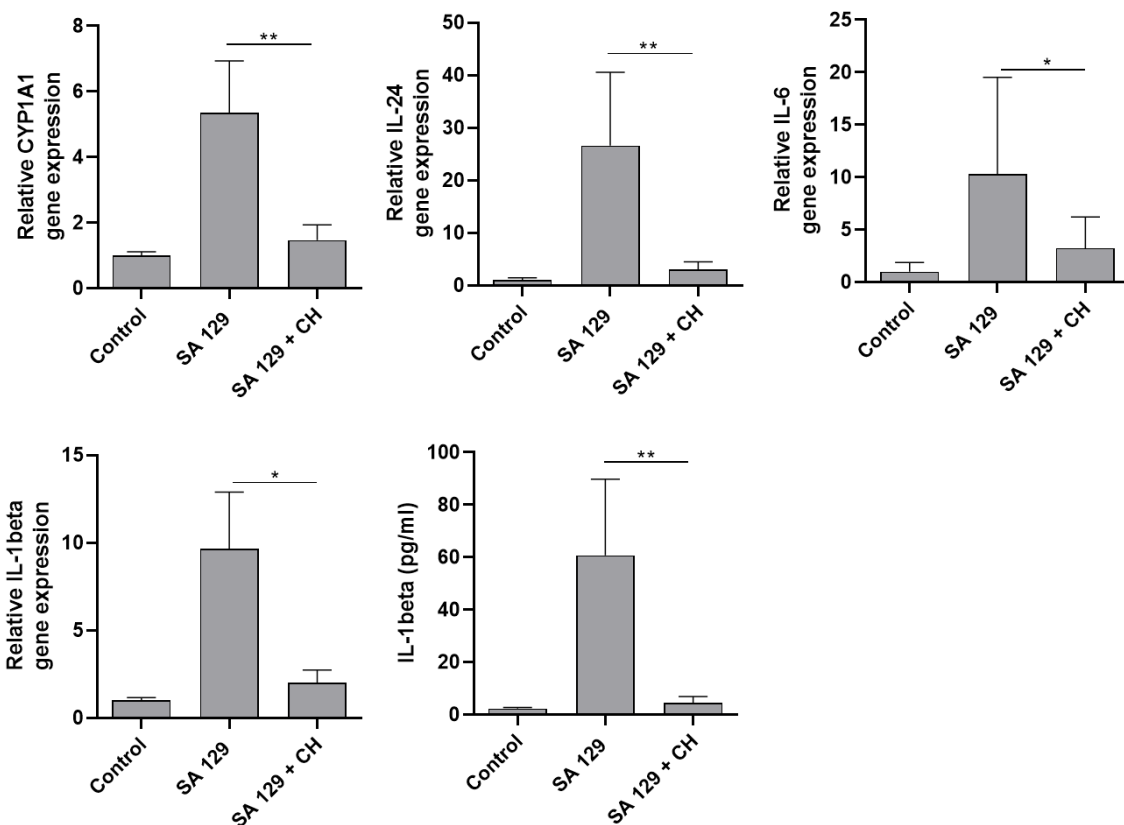
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241 Fig. 3 *S. aureus* induces AhR target gene expression in 3D skin equivalents. 3D skin equivalents were  
242 stimulated for 20-24 h with living *S. aureus* clinical isolate SA 129 in the presence or absence of the

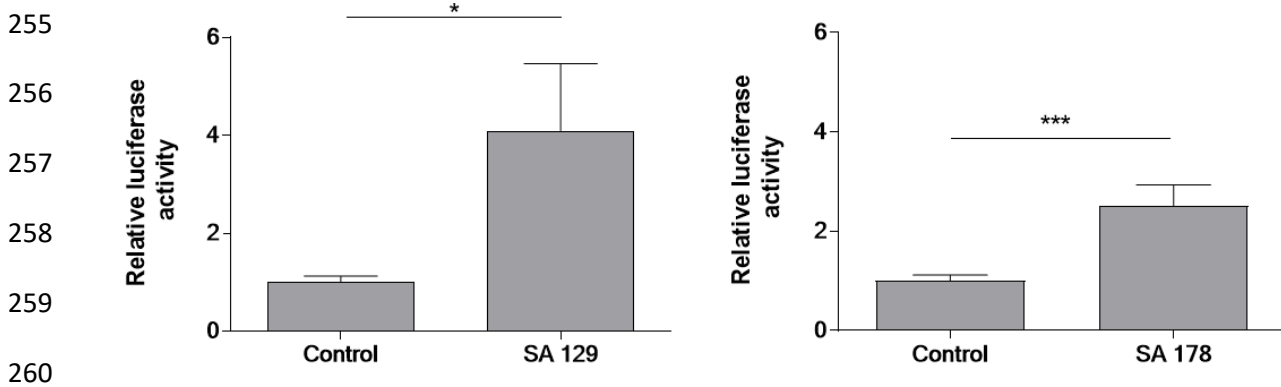
243 AhR inhibitor CH-223191 (CH). Gene expression of the AhR-responsive genes CYP1A1 and CYP1B1 as  
244 well as the cytokines IL-24, IL-6 and IL-1beta was analyzed by real-time PCR and shown as fold  
245 induction as compared to the unstimulated control. IL-1beta protein secretion was measured by  
246 ELISA. Shown are cumulative data of 5 skin equivalents (means + SEM; \*p < 0.05, \*\*p < 0.01).

247

248 *S. aureus* culture supernatants induce AhR target gene expression in primary keratinocytes

249 We next sought to determine whether the observed AhR-dependent *S. aureus*-mediated induction of  
250 AhR target genes was mediated by factor(s) released by *S. aureus*. To this end we transfected NHEKs  
251 with an AhR luciferase reporter plasmid and stimulated the cells with culture supernatants of *S.*  
252 *aureus* isolates SA 129 and SA178. This revealed an enhanced luciferase activity indicating activation  
253 of the AhR (shown in Fig. 4).

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261

262 Fig. 4. *S. aureus* culture supernatants induce AhR-luciferase reporter activity. NHEKs were  
263 transfected with an AhR *firefly* luciferase reporter plasmid (pGudLuc6.1) and a *renilla* luciferase  
264 control plasmid (hRLuc/TK). 48 h later the cells were stimulated with culture supernatants (1:5  
265 dilution) of *S. aureus* clinical isolates SA 129 and SA 178 for 16-18 h. AhR activation was determined  
266 by measuring luciferase activity, which was calculated as the ratio of *firefly* and *renilla* luciferase  
267 activities. Shown are means + SEM (n = 13 (SA 129) and n = 16 (SA 178); \*p < 0.05, \*\*\* p<0.001,  
268 Mann-Whitney-U test).

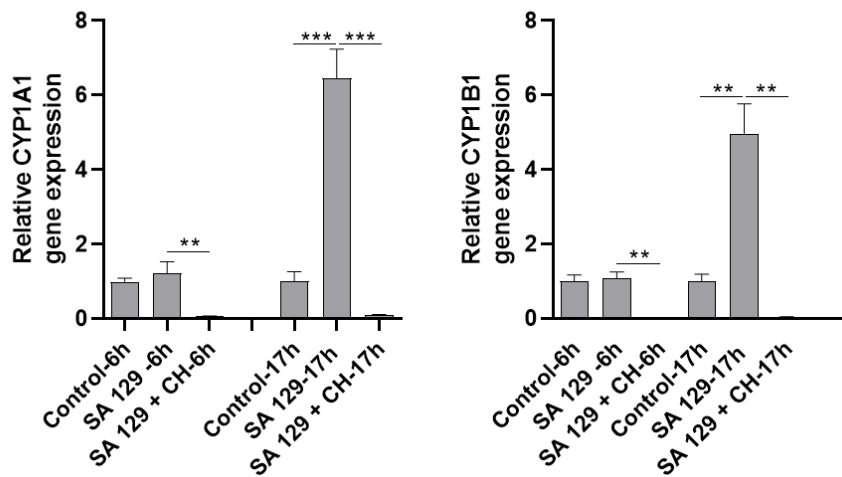
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270 Subsequently we stimulated NHEKS with culture supernatants of *S. aureus* SA 129 and SA 178 for 6 h  
271 and 17h and analyzed gene expression of the AhR responsive genes CYP1A1 and CYP1B1 by real-time  
272 PCR. Induction was seen only after 17 h (shown in figure 5A, B). Stimulation of the NHEKs with < 2

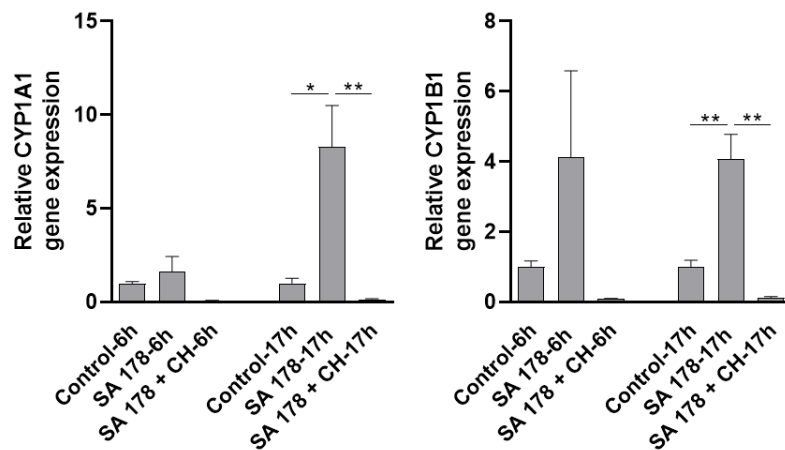
273 kDa and > 2 kDa ultrafiltrates of *S. aureus* culture supernatants revealed induction of CYP1A1 only  
274 with the < 2 kDa ultrafiltrate. This induction was blocked by CH-223191 (shown in figure 5c). These  
275 data indicate that the AhR-inducing activity is present in the < 2 kDa ultrafiltrate.

276 We next inhibited the expression of the AhR in NHEKs by transfection of the cells with an AhR-  
277 specific siRNA. This revealed a knockdown of AhR expression of 85% (shown in figure 5d). Stimulation  
278 of the AhR-siRNA-treated NHEKs with culture supernatant of *S. aureus* SA 178 revealed decreased  
279 induction of IL-24, IL-6 and L-1beta (shown in figure 5d). These data show that *S. aureus* secretes  
280 factor(s) that induce the cytokines IL-24, IL-6 and L-1beta in an AhR-dependent manner.

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B



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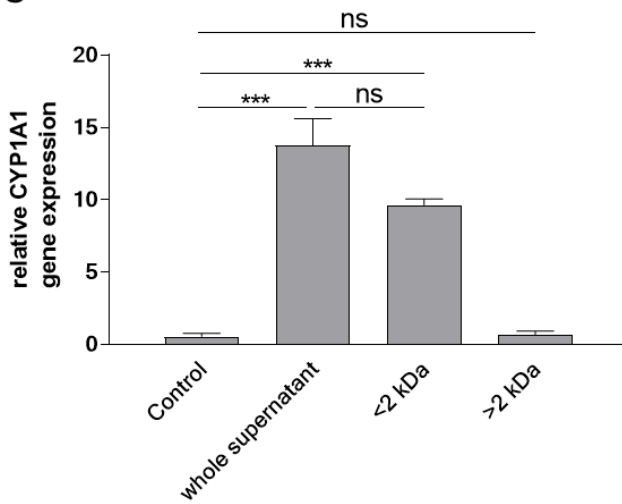
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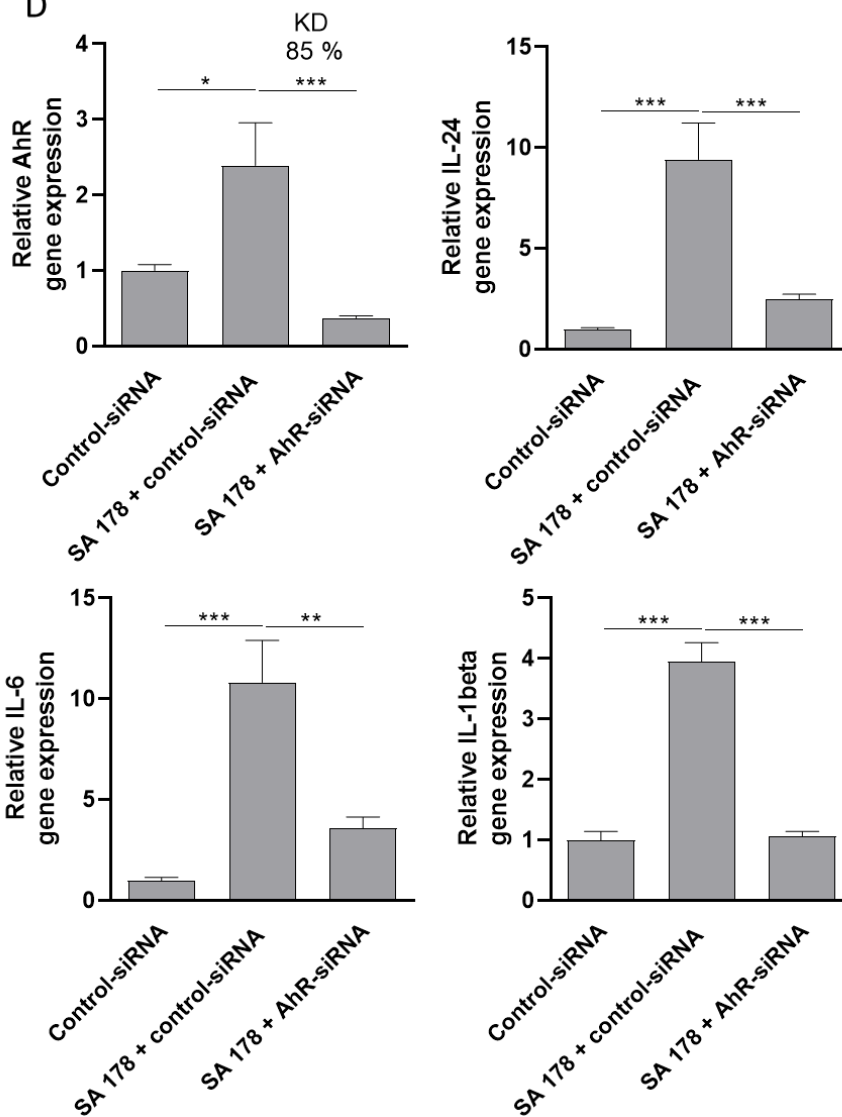
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D



307 Fig. 5: *S. aureus* culture supernatants induce AhR target gene expression. NHEKs were stimulated with  
308 culture supernatants of *S. aureus* isolates SA 129 (a) and SA 178 (b) for 6 h and 17 h with or without  
309 the AhR inhibitor CH-223191 (CH). (c) NHEKs were stimulated with culture supernatants of *S. aureus*  
310 isolates SA 178, either whole non-filtered supernatant or supernatant with a molecular weight < or >2  
311 kDa. (d) NHEKs were transfected with a control siRNA and an AhR-specific siRNA and stimulated with  
312 culture supernatants of SA 178. Relative gene expression was analyzed by real-time PCR. Bars are  
313 means + SEM of three (a-c) or six (d) stimulations (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

314

315

## 316 Discussion

317 The role of the AhR in cutaneous defense is still emerging. Recent studies reporting that bacteria  
318 such as *Pseudomonas aeruginosa* [10] and *Staphylococcus (S.) epidermidis* as well as *Malassezia*  
319 yeasts [16] activate the AhR strengthen the hypothesis that the AhR may serve as an ancient pattern  
320 recognition receptor. Moreover, a recent mouse study has shown that murine skin lacking AhR  
321 signaling displayed enhanced epidermal barrier defects. Interestingly, topical colonization with a mix  
322 of defined bacterial skin commensals (*S. epidermidis*, *S. hemolyticus*, *S. warneri*, *Micrococcus luteus*,  
323 *Corynebacterium aurimucosum*) restored epidermal barrier function. This study highlights an  
324 important role of the AhR in the epidermal barrier-microbiota interplay and provides further  
325 evidence of a crucial role of the AhR in bacterial sensing [24].

326 In the present study we show for the first time that the AhR is involved in the recognition of the  
327 important skin pathogen *S. aureus* by keratinocytes. Various *S. aureus* strains were able to induce  
328 expression of the AhR responsive gene CYP1A1 in keratinocytes indicating that *S. aureus* in general  
329 has the capacity to activate the AhR. Thus, the AhR may play a major role in the interplay of  
330 keratinocytes and *S. aureus* and may act as a pattern recognition receptor to sense the presence of *S.*  
331 *aureus*. Our data show that the AhR-activating factor(s) released by *S. aureus* has/have a molecular  
332 weight < 2 kDa which is in line with the characteristics of small aromatic hydrocarbons as AhR  
333 ligands. It is known that tryptophan metabolites act as ligands of the AhR [25] and such tryptophan-  
334 derived AhR ligands may be produced by *S. aureus*, a hypothesis that remains to be proven. A recent  
335 study showed that peptidoglycan, a bacterial ligand of Toll-like receptor-2 (TLR-2), led to increased  
336 CYP1A1 gene expression in keratinocytes indicating activation of AhR signaling. Given the size of

337 peptidoglycan, it is rather unlikely that it serves as a direct AhR ligand. Accordingly, the authors of  
338 that study assume that peptidoglycan may indirectly activate AhR signaling through stimulated  
339 production of endogenous AhR ligands [26].

340 There is increasing evidence that therapeutically targeting the AhR may ameliorate skin-associated  
341 inflammatory scenarios as seen in the chronic inflammatory skin diseases psoriasis and atopic  
342 dermatitis [27]. On the other hand, AhR expression is induced in psoriasis and atopic dermatitis [28]  
343 and mice constitutively overexpressing AhR in keratinocytes reveal a disturbed epidermal barrier and  
344 increased inflammation that resembled typical atopic dermatitis [29]. It has been hypothesized that  
345 under specific pro-inflammatory conditions AhR signaling might be compromised and thus  
346 restoration of AhR signaling by AhR agonist may offer a beneficial treatment strategy. In other  
347 conditions where an environmental over-activation of the AhR takes place, it would be preferable to  
348 dampen AhR signaling. This may also play a role in the prevention of skin cancer and skin aging [30].

349 We have shown that *S. aureus* induces IL-24 in keratinocytes, a process that required activation of  
350 the AhR. This implies that activation of the AhR in general may lead to increased IL-24 levels. In line  
351 with these data, AhR agonists increased IL-24 in an AhR-dependent manner in lung cells and thus IL-  
352 24 may contribute to the inflammatory effects of environmental AhR agonists [31]. Moreover, the  
353 AhR agonist tapinarof induced the secretion of IL-24 in keratinocytes and IL-24 negatively regulated  
354 expression of the skin barrier proteins filaggrin and loricrin [32]. Despite these inhibitory effects of  
355 tapinarof-induced IL-24 on filaggrin and loricrin, both proteins were surprisingly induced in  
356 keratinocytes treated with tapinarof [32]. IL-24 activated also the JAK1-STAT3 and MAPK pathways in  
357 keratinocytes and induced the secretion of pro-inflammatory mediators IL-8, PGE2, and MMP-1 [33].  
358 In transgenic mice that overexpressed IL-24 in the skin, abnormal epidermal differentiation and  
359 proliferation were observed accompanied by increased chemokine production and macrophage  
360 infiltration [34]. Accordingly, it has been suggested that topical tapinarof application may promote IL-  
361 24 expression by keratinocytes thus promoting skin inflammation [32]. Another study suggested that  
362 cytokines targeting the IL-20 receptors type I and II including IL-24 promote cutaneous *S. aureus*  
363 infection in a mouse model by downregulating IL-1beta and IL-17A dependent pathways. As  
364 mentioned in the introduction, increased susceptibility for cutaneous *S. aureus* colonization is  
365 associated with atopic dermatitis [4]. Interestingly, elevated IL-24 levels are present in the lesional  
366 skin of atopic dermatitis patients [35]. Moreover, a recent transcriptome study using skin biopsies  
367 revealed that AhR gene expression positively correlated with AD disease severity scores [21].  
368 Together, these data suggest that activation of the AhR by AhR agonists may trigger inflammatory  
369 processes by increased production of IL-24. Our results imply that activation of the AhR by *S. aureus*

370 may promote *S. aureus*-mediated inflammatory processes by increased AhR-dependent production  
371 of IL-24, a process that may be relevant in AD and other skin infections. Similarly, we also found an  
372 increased AhR-dependent induction of IL-6 and IL-1beta in *S. aureus*-treated keratinocytes. Both  
373 cytokines have been also implicated in the pathogenesis of AD. Thus, an AhR-mediated inflammatory  
374 response triggered by *S. aureus* may contribute to skin inflammation in AD. On the other hand, IL-  
375 1beta induces human beta-defensin (hBD)-2 in keratinocytes and hBD-2 protected against skin  
376 damage mediated by a *S. aureus* protease [36]. Therefore, the AhR-dependent IL-1beta induction by  
377 *S. aureus* may also have beneficial effects to control *S. aureus*-related harmful effects. Further studies  
378 are required to decipher the exact role of the AhR in atopic dermatitis and other inflammatory skin  
379 diseases.

380 In summary, our study highlights an important role of the AhR in sensing the important skin  
381 pathogen *S. aureus* by keratinocytes. This provides further evidence for the crucial role of the AhR in  
382 innate defense. Future studies have to show whether interference with cutaneous AhR signaling may  
383 offer therapeutic options to treat or prevent infectious skin diseases.

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## 392 **Conflict of Interest Statement**

393 The authors have no conflicts of interest to declare.

394

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400 **Author Contributions**

401 ELS, FR, RG and JH conceived and designed the experiments. ELS, FR, KAD, NH and LM performed the  
402 experiments and acquired the data. ELS, FR, LM, RG and JH analysed the data and prepared the  
403 figures. ELS,FR,RG and JH wrote the paper. All authors discussed the results and commented on the  
404 manuscript.

405

406 **Data Availability Statement**

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



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