Targeting the Cutaneous Microbiota in Atopic Dermatitis by Coal Tar via AHR-Dependent Induction of Antimicrobial Peptides

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Skin colonization by Staphylococcus aureus and its relative abundance is associated with atopic dermatitis (AD) disease severity and treatment response. Low levels of antimicrobial peptides in AD skin may be related to the microbial dysbiosis. Therapeutic targeting of the skin microbiome and antimicrobial peptide expression can, therefore, restore skin homeostasis and combat AD. In this study, we analyzed the cutaneous microbiome composition in 7 patients with AD and 10 healthy volunteers upon topical coal tar or vehicle treatment. We implemented and validated a Staphylococcus-specific single-locus sequence typing approach combined with classic 16S ribosomal RNA marker gene sequencing to study the bacterial composition. During coal tar treatment, Staphylococcus abundance decreased, and Propionibacterium abundance increased, suggesting a shift of the microbiota composition toward that of healthy controls. We, furthermore, identified a hitherto unknown therapeutic mode of action of coal tar, namely the induction of keratinocyte-derived antimicrobial peptides via activation of the aryl hydrocarbon receptor. Restoring antimicrobial peptide levels in AD skin via aryl hydrocarbon receptor–dependent transcription regulation can be beneficial by creating a (anti)microbial milieu that is less prone to infection and inflammation. This underscores the importance of coal tar in the therapeutic aryl hydrocarbon receptor armamentarium and highlights the aryl hydrocarbon receptor as a target for drug development.

INTRODUCTION

The human skin harbors many different bacteria, fungi, and viruses, which together constitute the cutaneous microbiome. The bacterial composition of healthy human skin varies between individuals, but, in general, Actinobacteria, Firmicutes, Proteobacteria, and Bacteriodetes are among the most abundant phyla (Grice et al., 2008). The microbiota composition is highly dependent on the anatomical site, and strongly relates to the level of moist and sebum present (Choi et al., 2012). Interestingly, the cutaneous microbiome is altered in highly prevalent chronic inflammatory skin diseases, such as psoriasis and atopic dermatitis (AD; Zeeuwen et al., 2013). It has been shown that the microbiome composition in lesional psoriatic skin differs largely from healthy skin (Alekseyenko et al., 2013; Assarsson et al., 2018; Fahlén et al., 2012; Gao et al., 2008; Loesche et al., 2018; Tett et al., 2017); however, many study results of these investigations are contradictory and have not led to the identification of a psoriasis-specific microbiome. For AD, on the other hand, the microbiome dysbiosis has been widely replicated, and it is now generally accepted that AD is strongly correlated to the colonization of the skin by Staphylococcus aureus, and that severity of AD lesions correlates with the relative level of S. aureus on the skin. Furthermore, S. aureus abundance is associated to disease flares and therapeutic responses (Higaki et al., 1999; Kong et al., 2012; Leyden et al., 1974), and S. aureus colonization can drive AD-like disease in mice (Kobayashi et al., 2015). A role for increased S. epidermidis colonization in AD has been reported (Byrd et al., 2017; Kong et al., 2012), but other findings imply a protective role for S. epidermidis in AD by producing antimicrobial peptides that selectively target S. aureus (Nakatsuji et al., 2017).

Conventional therapies for AD mainly target the immune system (e.g., corticosteroids or biologics), and aim at moisturizing the dry skin by topical use of emollients. The use of corticosteroids (e.g., fluticasone) and immunosuppressive drugs (e.g., tacrolimus) has shown to reduce skin inflammation and S. aureus colonization in AD (Gonzalez et al., 2016; Hung et al., 2007). One of the oldest topical treatments in

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Abbreviations: AD, atopic dermatitis; AHR, aryl hydrocarbon receptor; AMP, antimicrobial peptide; CT, coal tar; HC, healthy control; LCE, late cornified envelope; SLST, single-locus sequence typing

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The use of coal tar (CT), and this therapy is well-established in the management of various skin diseases, especially psoriasis and AD. The use of CT in AD is often accompanied by extensive remission periods, suggesting a long-term mechanism of action of the CT treatment regime. CT is a complex mixture of over 10,000 chemicals, including high concentrations of polycyclic aromatic hydrocarbons, which are known environmental pollutants and have been shown to affect the indigenous bacteria composition in soil (de Menezes et al., 2012; Yang et al., 2014; Zhang et al., 2010). Our group recently discovered the mechanism of action of CT during AD treatment. CT activates the aryl hydrocarbon receptor (AHR), leading to a restored skin barrier through induced keratinocyte differentiation (e.g., upregulated filaggrin and hornerin levels) and dampening of the T helper type 2 immune response in keratinocytes (van den Bogaard et al., 2013). Filaggrin contributes to the acidic mantle of the skin via its breakdown products known as natural moisturizing factors and is vital for providing a microenvironment in which antimicrobial peptides (AMPs) are active. CT-mediated induction of filaggrin may thus indirectly shape the microbiome by regulating the activity of keratinocyte-derived AMPs. Keratinocytes express many AMPs to control skin colonization by microbes. Notable examples are S100 calcium binding proteins (Gläser et al., 2005), human β-defensins (Harder et al., 1997), SKALP/ela-fin (Hiemstra et al., 1996), SLPI (Stetler et al., 1986), LL-37 (Zanetti et al., 1995), and the recently reported group of late cornified envelope (LCE) proteins (Niehues et al., 2017). Furthermore, a recent study showed that upon induced keratinocyte differentiation, protein breakdown products, in this case derived from hornerin, can function as antimicrobial peptides (Gerstel et al., 2018). This illustrates that CT may influence the cutaneous microbiota composition via elevating antimicrobial peptide levels in the stratum corneum. Others have shown that creams containing petrolatum induce keratinocyte differentiation and AMP expression (Czarnowicki et al., 2016). Therefore, we postulate that CT may alter the cutaneous microbiome composition, changing it toward that of healthy skin and, thereby, contributes to alleviating AD symptoms, adding a new therapeutic pillar to the mode of action of CT treatment. For this, we studied the effects of the CT treatment regime, similar to its use in daily clinical practice, on the bacterial composition of the human skin microbiome of healthy volunteers and patients with active AD lesions. We collected the microbiome by swabbing the antecubital fossa skin, and longitudinally determined the bacterial composition by 16S ribosomal RNA marker gene sequencing (16S, in short) and a single-locus sequencing typing (SLST) approach for in-depth analysis of the composition of the Staphylococcus genus down to the (sub)species level. Subsequently, we studied the expression of AMPs by CT treatment and delineated the key role for AHR signaling in this process.

RESULTS

Study cohort and baseline characteristics

We enrolled 17 human volunteers (10 healthy volunteers and 7 patients with AD) to study the effect of CT treatment on the composition of the cutaneous microbiome. Microbiome samples were taken from the antecubital fossa of healthy controls (HCs) and patients with AD, and bacterial composition according to 16S was measured over the course of 2 weeks, during treatment with either CT or control vehicle cream (Figure 1). To streamline longitudinal analysis, microbiome sampling days were grouped at 0, 4, 7, and 10 days after the start of the treatment. There were relatively more male individuals in the AD group (71.4%) in comparison with the group of HCs (30.0%; Table 1). Therefore, where applicable, statistical analyses were corrected for gender and age to avoid any potential confounding characteristics. To study the direct and indirect effect of CT on healthy skin, we enrolled 10 healthy volunteers for in vivo CT and vehicle treatment, and 10 healthy volunteers for in vivo tape stripping followed by CT and vehicle treatment.

**Propionibacterium in healthy skin and Staphylococcus in AD skin strongly associate with microbiome niches**

16S sequencing of the V3–V4 region (Zeeuwen et al., 2017) was used to measure the bacterial composition of healthy and diseased human skin (Supplementary Table S2). The bacterial composition at the genus level differed considerably between samples taken before the start of the treatment protocol, with similarity mainly determined by disease-status and, to a lesser extent, by the individual (Figure 2). In our cohort, healthy skin controls consisted, for a substantial part, of *Propionibacterium* spp. (69.6% ± 17.1% standard deviation), and only a relatively small proportion of *Staphylococcus* spp. bacteria (1.8% ± 1.8%). In concordance with other studies,
Staphylococcus was the most abundant genus-level taxon identified (47.6% ± 31.2%) in the microbiome of AD skin in our cohort, whereas Propionibacterium contributed to 16.2% ± 16.5% of this bacterial niche. In fact, Propionibacterium and Staphylococcus were found to be strongly anti-correlating (Spearman rho = -0.85, P < 0.0001), albeit this is likely a “communicating vessels” phenomenon. Supplementary Figure S1 shows microbiota compositional clustering of samples based on between-sample distances of all study samples (n = 134). A principal component analysis on all samples revealed that the largest differences between healthy and AD skin microbiota are explained by levels of Propionibacterium, Pseudomonas, and Corynebacterium (Supplementary Figure S2). The correlation of Staphylococcus abundance with the validated Eczema Area and Severity index illustrates the potential role of the Staphylococcus genus in AD pathogenicity or severity (Spearman rho coefficient of 0.71; Supplementary Figure S3).

These results are in line with current literature on AD microbiome composition (Chng et al., 2016; Higaki et al., 1999; Kong et al., 2012; Leyden et al., 1974) and validate the suitability of our study cohort to assess the effects of CT treatment on the microbiome composition of AD skin.

**CT and vehicle cream treatment alter the bacterial composition of healthy and AD skin**

Next, we analyzed the microbial composition of healthy and AD skin during treatment with CT cream and vehicle cream. Multivariate genus-level composition analysis shows that the application of CT, but also vehicle cream, altered the levels of several bacterial taxa in both healthy and AD skin (Figure 3a; HCs, green circles; AD, orange squares). During treatment period with CT cream, the microbial composition of AD skin shifted gradually, from day 0 to day 10, and eventually completed clinical remission, toward that of healthy skin, as shown by movement over treatment time to the left in principal component analysis space, of samples horizontally separated on condition (Figure 3b). During this period, we found a decrease in taxa belonging to the Staphylococcus genus (Figure 3c) in combination with an increase in the relative abundance of Propionibacterium (Figure 3d). A paired representation of Figure 3c and Figure 3d with corresponding paired statistics shows that these effects also persist on the intra-individual level (Supplementary Figure S4). These two genera contributed most strongly to the shift of an “AD microbiome” toward that of healthy skin. The effect of CT treatment in AD is borderline significant for Staphylococcus (P = 0.055; Figure 3c) and Propionibacterium (P = 0.095; Figure 3d), and vehicle treatment showed a similar trend, albeit less strong. In healthy skin samples, we found a significant decrease (P = 0.010) and a borderline significant decrease (P = 0.077) of Propionibacterium over 1-week course of CT and vehicle cream treatment, respectively (Figure 3e). In contrast, other bacterial families such as Enterobacteriaceae, Streptococcaceae, and Veillonellaceae increased in

![Figure 2. Cutaneous microbiota composition in HC and patients with AD.](image-url) Each leaf of the tree represents the start samples (taken at time-point 0) of both arms (averaged) of a single volunteer. Samples were clustered based on beta diversity (“between-sample distance”), using weighted UniFrac as a distance measure and hierarchical UPGMA as a clustering method. Horizontal bars show the relative abundance microbiota composition on the genus level, whereas reads that could not be classified up to this level are in white. The 11 most dominant genera are shown in the legend. AD, atopic dermatitis; HC, healthy controls; UPGMA, unweighted pair group method with arithmetic mean.
relative abundance on healthy skin during the first week of treatment, again irrespective of application of CT cream or vehicle cream (Figure 3f–h). Where in AD skin we see a shift from an AD microbiome toward that of healthy skin, which is in line with the complete clinical remission of the AD phenotype, the differences in healthy skin upon treatment are not accompanied by phenotypical alteration. The effects on the healthy skin microbiome are similar for CT and vehicle treatments, suggesting that other factors, such as AMP production or occlusion of the skin, might play a role.

**SLST as a method to analyze specific *Staphylococcus* species**

To further specify which *Staphylococcus* species are abundant on AD skin and which are changing during treatment, we applied SLST to a subset of our AD samples, with genus-specific primers targeting *Staphylococcus* taxa only (Ederveen et al., 2019). The SLST method allows for high-resolution profiling of a taxon of interest, *Staphylococcus* in this case, up to species level and beyond. This forms an asset to current 16S-based analyses, as 16S-based species level classification is notoriously difficult and suffers from low confidence (Kim et al., 2011).
In concordance with the 16S sequencing results, SLST revealed that the microbiome of lesional AD skin mainly consists of Staphylococcus species like S. aureus (34.3%, of total Staphylococci), S. capitis (29.9%), and S. epidermidis (7.1%; Figure 4). For legibility, Figure 4a is cropped from the full figure (Supplementary Figure S5). Although S. aureus and S. capitis are not significantly affected by CT treatment (Figure 4b), it is interesting to observe that treatment with CT significantly reduces many phylogenetically-related S. aureus—like and S. capitis—like taxa (n = 15 alleles [P = 0.04] and n = 16 alleles [P = 0.04], respectively; marked by green asterisk in Figure 4a, and, in more detail, represented as bar graphs in Figure 4b), whereas this is not observed for vehicle-treated skin. The increase in S. epidermidis, which is considered a health-associated commensal (Kloos and Musselwhite, 1975; Nakatsui et al., 2017), was much larger for CT treatment than for vehicle (3.4 fold-change and 0.3 fold-change, respectively). Although this effect was not statistically significant, likely because of interindividual variation, low relative levels, and a small sample size, the potential biological relevance may be of interest for further mechanistic investigations.

Finally, multivariate analysis of the SLST data provides similar conclusions as for the above-mentioned univariate contrast analysis. Mainly, 16S analysis on the same subset of AD samples, as selected for SLST, points to Staphylococcus associating with start conditions (i.e., active AD lesion), and Propionibacterium associating with treatment after 7 days, irrespective of CT or vehicle (Supplementary Figure S6a). Hence, these observations are in line with the presented data on the full study sample (Figure 2 and 3). When combining 16S and SLST data in one principal component analysis, Propionibacterium is found, again, associating with treatment after 7 days (irrespective of treatment type). An advantage of this combined 16S and SLST data is that relative abundances are corrected for Staphylococcus-levels according to the 16S dataset (i.e., the abundances are relative to the total bacterial population, not relative to only the Staphylococcus fraction). Interestingly however, in addition to Propionibacterium associating with treatment, the 17 of 20 taxa associating with active AD lesional skin are, again, exclusively among the aforementioned significantly differentially abundant S. aureus—like and S. capitis—like alleles (Figure 4a [marked by green asterisk] and Supplementary Figure S6b), which suggests that these are of biological relevance.

CT-mediated AHR activation induces AMPs in keratinocytes

To gain mechanistic insight, we studied potential direct and indirect effects of CT treatment on the microbiome composition. We investigated the indirect effects of CT via induction of keratinocyte-derived AMPs. Primary keratinocytes exposed to CT showed induction of several well-known AMPs, such as the LCE3 family, S100 family genes, and SKALP (Figure 5a). To pinpoint the key regulatory events that drive the AMP induction, we knocked down the molecular target of CT, namely the AHR and its nuclear partner, aryl hydrocarbon receptor nuclear translocator. This knockdown resulted in significantly less AMP induction, indicating that the canonical AHR signaling pathway is key in the CT-mediated AMP expression (Figure 5b). As additional proof of a general AHR involvement in AMP induction by keratinocytes, we showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin, a classical full AHR agonist, is able to induce AMP expression in keratinocytes, whereas a blockade of the AHR pathway by GNF351, a selective AHR antagonist, diminished the induction of most AMPs (Figure 5c). Since AMPs are generally produced by keratinocytes in the last living cells layers of the epidermis, we used organotypic human epidermal equivalents to validate these findings in stratified epithelial cultures. Primary keratinocyte-based epidermal equivalents showed induced expression of most AMPs upon CT treatment (Figure 5d). Protein expression of LCE3 and SKALP was induced by CT treatment, although the expression of hBD2 was not affected (Figure 5e). In addition, in vivo treatment of undamaged skin with CT cream induced the expression of LCE3, whereas vehicle cream (containing petrolatum) showed no induction (Figure 5f). hBD2 and SKALP expression was not influenced by vehicle nor CT treatment. In vivo treatment of skin that was depleted of stratum corneum by tape-stripping procedure—which results in a decreased barrier function—was followed by increased expression of LCE3, SKALP, and hBD2 upon treatment with CT cream for 4 days (Figure 5g). Nevertheless, this effect was also seen upon vehicle cream treatment. Taken together, our data suggest that CT treatment can control AMP expression, which could explain the shift in microbial composition we found during the treatment with vehicle and CT (Figure 3b).

Finally, we also investigated the potential direct effects of CT on the microbiome composition, as it was previously shown for polycyclic aromatic hydrocarbons—contaminate soil (de Menezes et al., 2012; Yang et al., 2014; Zhang et al., 2010). We found bactericidal (Supplementary Figure S7a) and bacteriostatic effects (Supplementary Figure S7b) by CT to relevant skin bacteria in vitro. Growth of all tested bacteria was inhibited by the CT distillate at 12.5 mg/ml or higher. The extrapolation of these in vitro results to the in vivo situation is, however, not straightforward because of (i) the unknown actual CT concentration that is released from the ointments, (ii) the effects of the vehicle cream on the antimicrobial properties of CT, and (iii) the “occlusion” effect of the bandages used in the in vivo study. We, therefore, analyzed in vivo the amount of (colony-forming) microbiota before and after 24 hours of topical vehicle and CT treatment in 10 healthy volunteers; similar to the microbiome study we performed above. In 9 of 10 healthy volunteers, bacteria could be cultured (at both aerobic and anaerobic culture conditions) before and after treatment period (Supplementary Figure S7c). These experiments indicate that petrolatum and CT may directly alter the growth and survival of bacteria on the skin, but also underline that it is not likely that CT treatment kills or inhibits all colony-forming cutaneous microbiota in vivo. This notion is strengthened by the observed shift in cutaneous microbiota composition in our initial dataset.

DISCUSSION

The human microbiome, including that of the skin, has been a subject of investigation for the past decade. The recent advances in high-throughput sequencing techniques and the initiation of the human microbiome project in 2008 greatly...
Figure 4. SLST heatmap showing significantly reduced abundance of *S. aureus*-like and *S. capitis*-like species during CT treatment. (a) Panel shows a cropped heatmap summarizing the *Staphylococcus*-specific SLST data from three patients with AD, treated either with CT or with vehicle cream, at day 0 and day 7 of the treatment period (for the full heatmap see Supplementary Figure S5). The phylogenetic tree on the left is based on a SLST allele sequences alignment with maximum likelihood—based clustering by FastTree. The alleles were built with an SDI threshold of 0.6, resulting in 45 SNP positions of the SLST marker gene sequences. Alleles for which a reference genome was available were named after this reference (e.g., *S. epidermidis*, VCU129), instead of having...
propelled the research capabilities and, as a consequence, has immensely improved our understanding of the human microbiota. Studies have described the composition of the cutaneous microbiome of healthy volunteers, which differs between individuals and between body sites (Costello et al., 2009; Grice et al., 2009). The microbiome of damaged or inflamed skin, whether or not related to psoriasis, AD, or other skin conditions, has been studied intensely and reviewed recently (Bjerre et al., 2017; Byrd et al., 2018; Langan et al., 2018; Zeeuwen et al., 2013). In our study we corroborate the observed discrepancy in cutaneous microbiome composition of HCs and patients with AD (Figure 2) that has been reported in literature repeatedly (Chng et al., 2016; Higaki et al., 1999; Kong et al., 2012; Leyden et al., 1974), and we provide additional data down to the species level using the SLST approach.

Our study was primarily focused on investigating the treatment effects of CT on the skin microbiome composition, and we found that CT treatment can alter the microbial composition of the lesional AD microbiome by decreasing the abundance of Staphylococcus species and increasing the abundance of Propionibacterium (Figure 3a–d). It is important to notice that a similar effect on Staphylococcus species was also present by applying mere vehicle cream, which consists of vaseline-lanette cream and subsequently applied zinc oxide paste. The vehicle formulation itself might influence bacterial growth or affect bacterial colonization of the skin. Reports on the effect of commonly used moisturizers on skin barrier repair and antimicrobial response have shown that these “inert” formulations can affect skin barrier repair and antimicrobial response (Czarnowicki et al., 2016). In addition, CT activates the AHR, which in turn regulates keratinocyte differentiation and dampens the inflammatory responses (van den Bogaard et al., 2013). The expression of AMPs, such as defensins and cathelicidins, is known to be downregulated in AD skin (de Jongh et al., 2005; Hata and Gallo, 2008; Ong et al., 2002). Our findings show that CT treatment induces AMP production via canonical AHR signaling (Figure 5). This further substantiates the hypothesis of an indirect antimicrobial response upon CT treatment and, altogether, opens a new venue for therapeutic targeting of the microbiome by induction of AMPs. Other reports have shown that zinc oxide might influence bacterial growth in a direct manner (Pasquet et al., 2014). In addition, after application of the CT creams and vehicle creams to the antecubital fossa, the creams were covered with bandages. This procedure could have a semi-occlusive effect on the skin, thereby promoting the growth of anaerobic bacteria, such as Enterobacteriaceae, Streptococcaceae, and Veillonellaceae species (Figure 3f–h). Furthermore, occlusion effects on skin, which are widely known to affect skin barrier integrity (Zhai and Maibach, 2002), can possibly cause an elevated penetration of CT through the stratum corneum and subsequently induce keratinocyte differentiation and AMP production. Another possible confounder in our experimental approach is the daily clinical practice protocol in which the bandages are only removed every other day, and the patients are asked to refrain from thorough washing of the treated skin. Bacteria that are directly affected or killed during the treatment period are in this way not removed from the skin, and as the 16S and SLST methods do not discriminate between dead or alive bacteria, no conclusions can be drawn regarding functionality of the shift in microbiome composition without any mechanistic follow-up studies.

We analyzed the bacterial microbiome by targeting the V3–V4 region of the 16S ribosomal RNA gene. The use of the 16S ribosomal RNA gene, and, more specifically, which region of this gene to target using a next generation sequencing approach, has been open for debate (Kong, 2016; Meisel et al., 2016; Zeeuwen et al., 2017). We chose to use the V3–V4 region of the 16S ribosomal RNA gene because this allows us to target most of the bacteria (including Propionibacterium species) comprising the cutaneous microbiome. The drawback of using either the V1–V3 or V3–V4 region approach is that both cannot confidently classify a large number of the different Staphylococcus species (Ederveen et al., 2019). For the V1–V3 region, this is 11%, although it is 1% using the V4 region (Meisel et al., 2016). Because Staphylococcus species play a significant role in the microbiome of lesional AD skin, we used SLST to confidently identify which Staphylococcus species are present in our dataset. The use of 16S sequencing combined with a SLST approach can be an important tool in future research to fully analyze and understand the role of Staphylococcus species inhabiting diseased skin. Furthermore, because the 16S and SLST amplicons can be pooled before sequencing, this combined approach is economically feasible.

Our data solidify our hypothesis on the potential additional therapeutic effect of CT treatment by targeting the cutaneous bacterial composition via AMP production. Nevertheless, more studies are required to address the causal relation between AMP profiles and the effects on the cutaneous microbiome composition. Finally, the implementation and validation of a combined approach of 16S and SLST sequencing in our cohort shows enormous potential for the field to study bacterial biodiversity focused on a single genus, as this approach requires less material, is far less expensive, and needs less complicated bioinformatics analysis than a metagenomics approach.
We showed in Figure 4 and Supplementary Figure S5 that the abundance of \( S. \) aureus—like and \( S. \) capitis—like species declines during CT treatment, which is interesting given the pivotal role of \( S. \) aureus in the AD skin microbiome. On the other hand, the \( S. \) aureus abundance did not change. One possible explanation could be that this specific clade of \( S. \) aureus is unaffected by treatment, remains unchanged, and thereby increases in relative sense because other...
Staphylococci decrease (i.e., a “communicating vessels” effect). Although the “classical” S. aureus strains do not decline upon CT treatment, our SLST and 16S data, together with the clinical remission over time, suggests that the S. aureus—like and S. capitis—like decline of these bacteria could be relevant to the clinical improvement of the AD phenotype. Nevertheless, mechanistic and functional data are desired to better understand the Staphylococcus strain differences we found in relation to the clinical outcome. Because of the overall focus of the research community on the role of S. aureus, the role of S. capitis during AD might be undervalued.

In conclusion, the herein presented study on the impact of CT treatment on the skin microbiome and AHR-dependent epidermal AMP induction sheds new light on this ancient, yet highly effective and safe therapy (Roelofsen et al., 2010). Considering the increasing body of evidence on skin microbiome involvement in the pathophysiology of inflammatory skin diseases, such as AD and psoriasis, the shift in microbiome composition by CT treatment could be one of its therapeutic pillars. The additional insights into the mode of action of CT are important for future drug development, as exemplified by the current development of Tapinarof (Smith et al., 2017), an AHR-targeting topical drug currently in phase 3 trials. This clearly warrants a further investigation of the microbiome-modulating properties of molecules that target the AHR in various barrier epithelia.

MATERIALS AND METHODS

Study design, approval, and patient inclusion criteria

Microbiome analysis. All patients with AD and HCs were selected according to the inclusion and exclusion criteria as described in Supplementary Text S1. Furthermore, patients with AD were asked to participate in our study when the following criteria were met: (i) active lesions of AD located at left and right antecubital fossa, and (ii) no history of treatment with antibiotics during the last 3 months. In total, we enrolled 17 volunteers (HCs, n = 10; patients with AD, n = 7) who were treated both with CT and with vehicle cream, and who were sampled at multiple time-points, as detailed below (see Figure 1).

In vivo treatment and tape stripping. All HCs were selected according to the inclusion and exclusion criteria, as described in Supplementary Text S1. For the in vivo treatment, we enrolled 10 healthy volunteers, and for the tape-stripping study, another 10 healthy volunteers were included. In advance of study start, medical ethical committee (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen) approval and individual written informed consent were obtained. The study was performed according to the Declaration of Helsinki principles. We, furthermore, refer to Table 1 for descriptive statistics, and to Supplementary Table S2 for detailing sample characteristics, sample sequence read statistics, and the 16S compositional matrix.

Data availability statement

Datasets containing the raw, unprocessed, 16S and SLST sequencing reads are publicly available for download at the European Nucleotide Archive database (http://www.ebi.ac.uk/ena/) under study accession number PRJEB27442 (ERP109520). The sequencing data is available in FASTQ-format, including corresponding metadata for each sample. For any additional information of the sequencing samples, metadata included, we refer to Supplementary Table S2.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: JPHS, THAE, JS, SAFTH, PLJMZ, EHB; Data Curation: THAE, SAFTH; Formal Analysis: JPHS, THAE; Funding Acquisition: JS, PLJMZ, EHB; Investigation: JPHS, GR, NJMB, IMJvV; Methodology: JPHS, THAE, JB, SAFTH; Project Administration: SAFTH, JS, EHB; Resources: MK; Software: THAE, JB, SAFTH; Supervision: JS, SAFTH, PLJMZ, EHB; Validation: JPHS, THAE; Visualization: JPHS, THAE; Writing - Original Draft: JPHS, THAE; Writing - Review and Editing: JPHS, THAE, JS, PLJMZ, EHB.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.06.142.

REFERENCES


SUPPLEMENTARY TEXT S1

Full inclusion and exclusion criteria

Any subject who meets any of the following criteria will be excluded from participation in this study. It should be noted that these criteria are written for healthy controls. Patients with AD have eczematous lesions and, therefore, this is not an exclusion criterion for this group.

- Body Mass Index greater than or equal to 35, or less than or equal to 18.
- Use of any of the following drugs within the last 6 months:
  - Systemic antibiotics (intravenous, intramuscular, or oral);
  - Oral, intravenous, intramuscular, nasal, or inhaled corticosteroids;
  - Cytokines;
  - Methotrexate or immunosuppressive cytotoxic agents;
  - Large doses of commercial probiotics consumed (greater than or equal to 10^8 colony-forming units or organisms per day), including tablets, capsules, lozenges, chewing gum, or powders in which probiotic is a primary component. Ordinary dietary components, such as fermented beverages and/or milks, yogurts, foods, do not apply.
- Use of topical antibiotics, antifungal, or topical steroids within the previous 7 days.
- Acute disease at the time of enrolment (defer sampling until subject recovers). Acute disease is defined as the presence of a moderate or severe illness with or without fever.
- Chronic, clinically significant (unresolved, requiring ongoing medical management or medication) pulmonary, cardiovascular, gastrointestinal, hepatic, or renal functional abnormality, as determined by medical history or physical examination.
- History of cancer, except for squamous or basal cell carcinomas of the skin that have been medically managed by local excision.
- Unstable dietary history, as defined by major changes in diet during the previous month, where the subject has eliminated or significantly increased a major food group in the diet.
- Recent history of chronic alcohol consumption defined as more than five 1.5-ounce servings of 80 proof distilled spirits, five 12-ounce servings of beer, or five 5-ounce servings of wine per day.
- Confirmed positive test for HIV, HBV, or HCV.
- Any confirmed or suspected condition and/or state of immunosuppression or immunodeficiency (primary or acquired), including HIV infection.
- Major surgery of the gastrointestinal tract, with the exception of cholecystectomy and appendectomy, in the past 5 years. Any major bowel resection at any time.
- History of active uncontrolled gastrointestinal disorders or diseases including the following:
  - Inflammatory bowel disease, including ulcerative colitis (mild, moderate, or severe), Crohn's disease (mild, moderate, or severe), or indeterminate colitis;
  - Irritable bowel syndrome (mild to severe);
  - Persistent, infectious gastroenteritis, colitis, or gastritis, persistent or chronic diarrhea of unknown etiology, Clostridium difficile infection (recurrent), or Helicobacter pylori infection (untreated);
  - Chronic constipation.
- Female who is pregnant or lactating.
- Treatment for or suspicion of ever having had toxic shock syndrome.
- History of psoriasis or recurrent eczema.
- History of recurrent rashes within the past 6 months.
- At the time of the screening visit or on the specimen collection day:
  - Acne at sites other than on the face, chest, back, or shoulders;
  - Multiple blisters, pustules, boils, abscesses, erosions, or ulcers on the scalp, face, neck, arms, forearms, or hands;
  - A single blister, pustule, boil, abscess, erosion, ulcer, scab, cut, crack, or pink and/or hyperpigmented patch or plaque at or within 4 cm of the sampling sites; sampling may be deferred until the lesion resolves either without treatment or with local treatment only;
  - More than one pink and/or red scaly patch and/or plaque anywhere on the body (suggestive of psoriasis or eczema);
  - Uniformly thickened, cracking, “dry” skin on bilateral palms and/or soles;
  - Scalp dandruff that does not clear up with over-the-counter dandruff shampoos used daily for 2 weeks;
  - Disseminated rash (at multiple body sites or extending throughout a broad body area).
- Chronic dry mouth.
- Periodontitis and/or gingivitis.
- Evidence of untreated cavitated carious lesions or oral abscesses.
- Evidence of precancerous or cancerous oral lesions.
- Evidence of oral candidiasis.
- More than eight missing teeth. The missing teeth must be because of third molar extractions and/or teeth extracted for orthodontic purposes, teeth extracted because of trauma, or teeth that are congenitally missing.

Study procedures

- Subjects have to refrain from swimming in a chlorinated pool or using a hot tub for 48 hours before sampling visit.
- Subjects have to avoid the use of sauna and/or steam baths for 48 hours before sampling visit.
- Subjects have to avoid the use of tanning bed for 48 hours before sampling visit.
- Bathe and/or shower procedure:
  - Do NOT shower on the sampling day.
- Avoid the use of body lotion on the sampling day.

SUPPLEMENTARY MATERIALS AND METHODS

Coal tar (CT) cream formulations and treatment protocols

Vehicle creams. Vaseline-lanette (catalog number 102058; Fagron NL BV, Capelle aan den IJssel, The Netherlands) and zinc oxide paste (catalog number 98168312; Department of
Clinical Pharmacy and Pharmacology, UMCG, Groningen, The Netherlands).

CT creams. Both based on the vehicle creams used, with an addition of CT solution (10% liquor carbonis detergens; Fagron NL BV) in the vaseline-lanette cream and a 5% CT (Pix lithanthracis; Fagron NL BV) addition to the zinc oxide paste.

Microbiome analysis. Upon inclusion of the patients with atopic dermatitis, the Eczeema Area and Severity Index score was assessed as a standardized measure of atopic dermatitis severity at the start time-point. In all healthy controls and patients with atopic dermatitis, one antecubital fossa was treated with the vehicle cream (i.e., on one inner elbow), and the other was treated with the CT cream (randomly selected). The vehicle creams were applied subsequent to each other. First, the vaseline-lanette cream was applied to the antecubital fossa. Zinc oxide paste was applied on top. The CT creams were both based on the vehicle creams used, with an addition of CT solution on both creams. After application of the ointments, the treated arms were covered with standard medical bandage. The treatment creams were self-applied twice daily and the bandages were replaced every other day.

Tape-stripping experiments. A 4 mm punch biopsy was taken of non-tape-stripped skin, from the lower back, as start control. Subsequently, 3 × 1 cm² of skin was tape-stripped by continuous application and removal of tape until full removal of the stratum corneum. All three locations were either (i) untreated, (ii) vehicle cream-treated, or (iii) CT-treated for four consecutive days (self-applied treatment twice daily). Punch biopsies for further analysis were taken from all three locations after 4 days. During the complete study, all healthy volunteers (n = 10) were under medical supervision of a fully trained, experienced dermatologist.

24-hour treatment experiments. Bacterial swabs from both antecubital forearms of 10 healthy volunteers were collected and plated on BD Columbia III agar with 5% sheep blood plates (Becton Dickinson GmbH, Heidelberg, Germany) before treatment protocol. One antecubital forearm was treated with vehicle cream and the other was treated with CT cream for 24 hours. Afterwards, bacterial swabs were collected from both arms. Colony-forming units were allowed to grow in either aerobic or anaerobic conditions for at least 24 hours. Photographs were taken from plates after 24-hour incubation period.

Skin microbiome sample collection
All healthy controls cooperated according to the following sample collection scheme: sample collection at day 0, 4, and 7. The day 0 samples (“start” samples) were collected before the first application of the creams. Because of the “real-life clinical practice” character of the study, the days at which samples were collected from the patients with atopic dermatitis were not strictly enforced. All patients “start” samples were collected at day 0, before the first cream application. The other samples were collected at day 4, 7, 10 (± 1 day), and at the time-point of complete clinical remission, as assessed by the dermatologist. Sample collection was performed as described previously (Zeeuwen et al., 2012; Zeeuwen et al., 2017b).

In short, a 4-cm² skin area, located at the antecubital fossa, was swabbed with sterile Catch-All sample collection swabs (Epicentre Biotechnologies, Madison, WI). The swabs were soaked in sterile SCF-1 solution (50 mM Tris buffer (pH 8), 1 mM EDTA, and 0.5% Tween-20) before sample collection. Mock swabs, exposed to ambient air, were taken as negative controls. The Mobio UltraClean Microbial DNA isolation kit (Mobio Laboratories, Carlsbad, CA) was used according to the manufacturers protocol to extract microbial genomic DNA, before storing it at −80°C until further use.

16S ribosomal RNA gene and single-locus sequence typing (SLST) marker gene library preparation
To generate PCR amplicon libraries for microbiota sequencing, sample-specific amplicons were generated in-house by PCR with primers targeting (i) the V3–V4 hyper-variable region of the 16S small subunit ribosomal RNA gene for 16S sequencing, and (ii) the 30S ribosomal protein S11 gene of Staphylococcus for SLST sequencing. The Staphylococcus-specific SLST target was identified with the TaxPhIAm SLST-design bioinformatics pipeline and validated as described (Ederveen et al., 2019). In short, single-copy orthologous gene clusters of 200 representative Staphylococcus genomes were screened for suitable SLST regions, that is, having sufficient conservation for primer design and ample variation for discrimination of different Staphylococcus (sub) species. For 16S, 10–25 ng of genomic DNA was used as a template for the first PCR with a total volume of 50 µl using 357F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTT ACG GGA GGC AGC AG-3') and 802RV2 (5'-GTC TCG TGG GCT CGG AGA TGA GTA AAG AGA GTA CNV GGG TAT CTA AKC C-3') primers appended with Illumina adaptor sequences (Zeeuwen et al., 2017a). The italicized sequence represents the flag sequence, and the bold sequence represents the marker gene primer sequence. For SLST, likewise, 10–25 ng of genomic DNA was used as a template for the first PCR with a total volume of 50 µl using 1123F (5'-TCG TCG GCA GGC TCA GAT GTG TAT AAG AGA CAG CTT ACG GGA GGC AGC AG-3') and 802RV2 (5'-GTC TCG TGG GCT CGG AGA TGA GTA AAG AGA GTA CNV GGG TAT CTA AKC C-3') primers appended with Illumina adaptor sequences. Before sequencing, 16S and SLST amplicons were pooled 1:1 (v:v) and sent to the sequencer provider as further described below.

16S ribosomal RNA gene and SLST marker gene sequencing
Illumina 16S and SLST amplicon libraries were barcoded by an additional PCR (12 cycles), multiplexed, and sequenced at BaseClear BV (Leiden, The Netherlands) on an Illumina MiSeq paired-end 300 cycles system. The sequencing run was analyzed with the Illumina CASAVA pipeline (version 1.8.3), with demultiplexing based on the sample-specific barcodes. The raw sequencing data produced was processed removing the sequence reads of too low quality (only “passing filter” reads were selected) and discarding reads containing adaptor sequences or failing PhiX Control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FASTQC quality control tool, version 0.10.0. Multiplexed FASTQ files as provided by BaseClear were first used to generate Illumina
paired-end sequence pseudoreads by PEAR (Zhang et al., 2014), using the default settings. Thereafter, 16S and SLST reads were split by a custom in-house Python script based on a Basic Local Alignment Search Tool of the sequencing reads against our Staphylococcus SLST database.

16S sequencing data analysis (microbiota)
For 16S marker gene sequencing analysis, a customized Python workflow based on Quantitative Insights Into Microbial Ecology (version 1.8; Caporaso et al., 2010b) was adopted (http://qiime.org). Reads were filtered for chimera sequences using the UCHIME algorithm version 4 (Edgar et al., 2011). Hierarchical clustering of samples was performed using unweighted pair group method with arithmetic mean with weighted Unifrac as a distance measure, as implemented in Quantitative Insights Into Microbial Ecology 1.8. The Ribosomal Database Project (Wang et al., 2007) classifier, version 2.3, was performed for taxonomic classification of the sequence reads. Alpha diversity metrics (PD whole tree, Chao1, Observed Species, and Shannon) were calculated by bootstrapping 1768 reads per sample and taking the average. Beta diversity indices (Unweighted Unifrac, weighted Unifrac, Bray-Curtis dissimilarity score with the program NEIGHBOR of the PHYLIP package, version 3.6) were calculated. The resulting Staphylococcus alleles phylogenetic tree with samples heat map visualization was generated with interactive tree of life tool (Letunic and Bork, 2007). Note that because of technical limitations in the resolution of 16S marker gene sequencing, operational taxonomic unit calling on the level of species should be interpreted with caution.

SLST sequencing data analysis (Staphylococcus-specific)
Oligotyping of the SLST marker gene sequences was performed by the TaxPhlAn SLST-analysis bioinformatics pipeline (Ederveen et al., 2019). In short, SLST reads and SLST references were super-aligned by PYNAST (default settings; Caporaso et al., 2010a), and informative single-nucleotide polymorphism positions were determined by Shannon diversity index; a Shannon diversity index of 0.6 or greater was required to call a single-nucleotide polymorphism. Hereafter, Staphylococcus SLST alleles were built into a maximum-likelihood phylogenetic tree by FastTree (default settings; Price et al., 2009). Clustering of samples by Staphylococcus profiles was based on neighbor-joining of the Bray-Curtis dissimilarity score with the program NEIGHBOR of the PHYLIP package, version 3.6 (default settings). The resulting Staphylococcus alleles phylogenetic tree with samples heat map visualization was generated with interactive tree of life tool (Letunic and Bork, 2007).

Primary keratinocyte isolation
Human abdominal or breast skin was obtained from plastic surgery procedures after informed consent and in line with the principles and guidelines of the Declaration of Helsinki. Skin biopsies were taken and human primary keratinocytes were isolated as previously described (Tjabringa et al., 2008) and stored in liquid nitrogen until further use.

Submerged keratinocyte culture
Human primary keratinocytes were cultured in a 24-well plate in keratinocyte growth medium (Lonza, Walkersville, MD) until near 100% cell confluence before stimulation with CT (Pix lithanthracis in DMSO, final concentration 2 µg/ml; Fagron NL BV), 2,3,7,8-tetrachlorodibenzo-p-dioxin (10 nM; AccuStandard, New Haven, CT), or GNF351 (500 nM; Merck, Kenilworth, NJ). Cells were harvested 48 hours after stimulation for quantitative gene expression analysis.

Small interfering RNA knockdown
Keratinocytes were grown to 60% confluence, and 500 nM of smartpool (small interfering aryl hydrocarbon receptor and small interfering aryl hydrocarbon receptor nuclear translocator) or nontargeting small interfering RNA (Acell Dharmaco, Thermo Scientific, Waltham, MA) was added for 48 hours. Culture medium was subsequently refreshed and supplemented with small interfering RNA for another 24 hours. Thereafter, keratinocytes were allowed to differentiate for 48 hours in the presence of CT (2 µg/ml). Cells were harvested for transcriptional analysis as described below.

Human epidermal equivalent generation
Human epidermal equivalents were generated as previously described (Niehues et al., 2016; Nygaard et al., 2015). Briefly, inert transwells (ThinCerts, Greiner Bio-One GmbH, Kremsmünder, Austria) were coated with rat-tail collagen (100 µg/ml; BD Biosciences, Bedford, MA) at 4 °C for 1 hour. A total of 1 × 105 primary keratinocytes were seeded on the transwells in 100 µl CnT-prime (CellnTec, Bern, Switzerland) in a 24-well format. After 48 hours, cultures were switched to a mixture of CnT-PR-3D medium (CellnTec) and DMEM medium (60:40 [v/v]) for 24 hours, and then cultured at the air-liquid interface for 8 days. Culture medium was refreshed every other day. CT was supplemented (2 µg/ml) to the CnT-PR-3D medium for 72 hours before harvesting.

Histology and immunohistochemistry
Human epidermal equivalent constructs and in vivo biopsies were fixed in 4% formalin solution for 4 hours and subsequently embedded in paraffin. Sections of 6 µm were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO) or processed for immunohistochemical analysis. Sections were blocked for 15 minutes with 5% normal goat, rabbit, or horse serum in phosphate buffered saline, and subsequently incubated for 1 hour at room temperature with the following antibodies: mouse anti-LCE3 (Abmart, Berkeley Heights, NJ), goat anti-hBD2 (ab9871; Abcam, Cambridge, United Kingdom), or rabbit anti-SKALP (Vandermeeren et al., 2001). Next, a 30-minute incubation step with biotinylated horse anti-mouse, rabbit anti-goat, or goat anti-rabbit (Vector Laboratories, Burlingame, CA) was performed, followed by a 30-minute incubation with avidin-biotin complex (Vector laboratories). The peroxidase activity of 3-Amino-9-ethylcarbazole was used to visualize the protein expression and the sections were mounted using glycerol gelatin (Sigma-Aldrich).

Total RNA isolation and quantitative reverse transcriptase in real-time PCR
Total RNA was isolated using the Favorprep total tissue RNA kit (Favorgen Biotech, Taiwan), according to the manufacturer’s protocol. cDNA was generated, after DNase treatment, and used for quantitative reverse transcriptase in real-time PCR with the MyiQ Single-Color Real-Time Detection
Minimum inhibitory concentration analysis and callus model with CT distillates

Pure pix lithanthracis (Fagron NL BV) was dissolved in DMSO to a final concentration of 500 mg/ml. Several cutaneous bacteria species (S. aureus, S. epidermidis, S. capitis, S. hominis, Propionibacterium acnes, and Corynebacterium aurimucosum) were treated with a dilution series of CT distillates for 24 hours in a 96-well plate (liquid-culture minimum inhibitory concentration assay). Subsequently, all wells were diluted 10, 100, and 1000 times, and plated on BD Columbia III agar with 5% sheep blood plates (Becton Dickinson GmbH) and allowed to grow, either in aerobe or anaerobe conditions (<0.01% O2). After 24 hours, colony-forming units were counted and the minimum inhibitory concentration value was calculated as the concentration of CT where less than 1% of initial bacteria have survived. The callus experiments were performed as published before (van der Krieken et al., 2016). In short, bacterial growth was analyzed by adding a concentration range of CT distillates on top of the callus model for 24 hours. After diluting and plating the bacteria on BD Columbia III plates, colony-forming units were counted, and bacterial growth was calculated from these counts.

Statistical analysis

For the microbiota data, statistical significance between contrasts with regard to taxonomy abundances was tested by a non-parametric (unpaired) Mann-Whitney U test, uncorrected unless stated otherwise. Redundancy analysis and principal component analysis were done using Canoco 5.04 (ter Braak and Smilauer, 2012) using default settings of the analysis type “Constrained” or “Unconstrained”, respectively. In addition, log transformation was set to 100, unless stated otherwise. To prevent undesired biases in the multivariate redundancy and principal component analyses, age and gender were always taken along as a covariate. Relative abundance values for taxa were used as response data, and in case of redundancy analysis, the sample status as explanatory variables. Redundancy analysis calculates P-values by randomly permuting the sample status, and by, thereafter, counting the number of times that a permuted set of samples had a better separation than the original one. Data from the in vitro experiments are represented as mean ± standard error of the mean of at least three biological replicates. Raw ΔCt values were used to statistically analyze the quantitative PCR results using the commercially available GraphPad Prism software, version 8.1.1. Two-way analysis of variance followed by Bonferroni post hoc testing was performed.

SUPPLEMENTARY REFERENCES


Supplementary Figure S1. Clustering of all 134 study samples. Each leaf of the tree represents a single sample. Samples were clustered based on beta diversity (“between-sample distance”), using weighted UniFrac as a distance measure and hierarchical UPGMA as a clustering method. Vertical bars show the relative abundance microbiota composition on the genus level; reads that could not be classified up to this level are in white. UPGMA, unweighted pair group method with arithmetic mean.
Supplementary Figure S2. Principal component analysis of HC and AD samples based on their microbiota composition. The horizontal axis, explaining 29.5% of the variation between samples, partly separates HC samples from AD samples mainly based on the abundance of *Staphylococcus* and *Propionibacterium*. The vertical axis, explaining 17.2% variation, does not contribute to the separation of the sample groups, and, therefore, represent genera that do not associate with health or disease phenotype. Green circles and orange squares correspond to the centroids of HC volunteers and AD volunteers, respectively. Spearman Rho coefficient $-0.85$, $P < 0.0001$. AD, atopic dermatitis; HC, healthy control.

Supplementary Figure S3. Correlation between *Staphylococcus* and disease severity. The abundance of *Staphylococcus* of the AD samples at the start of the treatment period directly correlates to the EASI with a Spearman rho coefficient of 0.71. AD, atopic dermatitis; EASI, Eczema Area and Severity Index.

Supplementary Figure S4. Paired analysis of *Staphylococcus* and *Propionibacterium* in AD during treatment with CT or vehicle. This figure shows a paired representation of main figures 3c and 3d with corresponding paired statistics by paired MWU, that is, a Wilcoxon signed rank test. These results indicate that the observed overall dynamics of the microbiome during treatment of patients with AD with vehicle (a, b) or CT (c, d), that is, a decrease in *Staphylococcus* (Figure 3c) and an increase in *Propionibacterium* (Figure 3d), also persist on the intra-individual level. Note that paired data was only available for a subset of the individuals ($n = 3$ to $4$). AD, atopic dermatitis; CT, coal tar; MWU, Mann-Whitney U.
Supplementary Figure S5. SLST heatmap showing significantly reduced abundance of *S. aureus*-like and *S. capitis*-like species during CT treatment. The figure shows the total heatmap summarizing the Staphylococcus-specific SLST data from three patients with AD, either treated with CT or with vehicle cream, at day 0 and day 7 of the treatment period. The phylogenetic tree on the left is based on an SLST allele sequences alignment with maximum likelihood-based clustering by FastTree. The alleles were built with an SDI threshold of 0.6, resulting in 45 SNP positions of the SLST marker gene sequences. Alleles for which a reference genome was available were named after this reference (e.g., *S. epidermidis* VCU129), instead of having an allele number (e.g., allele #30452). Multiple references were collapsed into one clade if they shared the same allele sequence (e.g., *S. epidermidis*). The heatmap represents log10-transformed SLST relative abundances, not corrected for *Staphylococcus* genus-level abundances according to 16S. A green asterisk indicates significantly differentially abundances, according to a non-parametric MWU test, and corrected for *Staphylococcus* genus-level relative abundance derived from 16S data. Note that the phylogenetic tree was cropped at top and bottom to facilitate visualization, leaving out some lowly abundant and statistically insignificant taxa from this view (for a full list see Supplementary Table S3). AD, atopic dermatitis; CT, coal tar; MWU, Mann-Whitney U; SDI, Shannon diversity index; SLST, single-locus sequence typing; SNP, single-nucleotide polymorphism; V, vehicle treatment; T, CT treatment.
Supplementary Figure S6. PCA analysis of 16S and combined 16S and SLST data of AD samples. (a) The figure shows that start samples (centroid depicted by light blue square) and treatment samples after 7 days, either with vehicle cream or CT cream (centroids in dark blue and purple, respectively), differ mainly in abundance of *Propionibacterium*, *Pseudomonas*, and *Staphylococcus*. (b) The “combined” 16S and SLST data implicates that for each sample the original 16S data has been appended by available SLST data with regard to *Staphylococcus* (sub)species information. Start samples, n = 6; Vehicle and Tar samples, both n = 3. AD, atopic dermatitis; CT, coal tar; PCA, principal component analysis; SLST, single-locus sequence typing.
Supplementary Figure S7. CT distillates can directly affect bacterial survival and bacterial growth, but it is not likely to kill all cutaneous microbiota. CT distillates can act (a) bactericidal in a liquid-culture assay (MIC = 12.5 mg/ml, CT in DMSO) and (b) bacteriostatic on several relevant skin bacteria in the callus model. Growth of all tested bacteria was inhibited by the CT distillate at 12.5 mg/ml or higher. (c) The amount of (colony-forming) microbiota before and after 24 hours of topical vehicle and CT treatment (of the antecubital fossa, under occlusion). Only aerobic culture conditions are shown, given that *Staphylococcus* and *Corynebacterium* species (S7a and S7b) are grown under aerobic culture conditions. CT, coal tar; MIC, minimum inhibitory concentration.
**Supplementary Table S1. Primer Sequences**

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