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Differential induction of innate defense antimicrobial peptides in primary nasal epithelial cells upon stimulation with inflammatory cytokines, Th17 cytokines or bacterial conditioned medium from *Staphylococcus aureus* isolates

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ABSTRACT

To date it is incompletely understood why half of the human population is intrinsically resistant to *Staphylococcus aureus* colonization whereas the other half is intermittently or permanently colonized. Nasal colonization represents the primary niche for *S. aureus*. We therefore investigated whether primary nasal epithelial cells (HNEC) express antimicrobial peptides (AMPs) upon stimulation by inflammatory cytokines or bacterial conditioned medium (BCM) of different colonizing and invasive staphylococci. Stimulation with classical cytokines (IL-1β, TNF-α, IFN-γ) potently induced hBD-3 and RNase7 in HNEC. Th17 cytokines (IL-17A, IL-17F, IL-22) yielded comparably weak hBD-3 and RNase7 induction and no synergistic effects with classical cytokines. BCM of *S. aureus* and *Staphylococcus epidermidis* isolates moderately induced hBD3 and RNase7 mRNA expression without significant differences when comparing colonizing vs. invasive isolates. Our results indicate that HNEC contribute to the innate defense by secretion of an AMP-containing chemical defense shield along the nasal mucosa i.e. within the primary colonization niche of *S. aureus*. Further studies are needed to investigate whether a deficient AMP expression in the nasal mucosa may be related to different *S. aureus* carrier states. AMPs or AMP-inducing agents may be promising candidates for future topical decolonization regimens that aim to prevent invasive *S. aureus* infections.

1. Introduction

Staphylococcus aureus is known to be both, a commensal of the human nose but also a pathogen, leading to invasive infections ranging from superficial skin and soft tissue infections to deep-seated metastatic or disseminated infections. Longitudinal studies revealed that 20% of healthy adults are permanent carriers, 30% are intermittent carriers as opposed to 50% non-carriers that seem to be 'intrinsically-resistant' to *S. aureus* colonization. Although multiple body sites like the perineum, axilla, the skin of the trunk, palms and soles as well as the gastrointestinal tract, vagina and pharynx may be colonized to varying extents, the nares represent the primary niche and principal carriage site of *S. aureus* in human beings [1].

To date, neither the virulence factors of *S. aureus* nor the contribution of the innate immune defense within the host that lead to the observed colonization patterns are completely understood. In contrast it is well established that *S. aureus* carriage represents a risk factor for community-acquired skin and soft tissue infections [1]. Moreover, the higher bacterial count in the nose observed in persistent carriers may render these patients particularly prone to nosocomial or healthcare-associated *S. aureus* infections [2]. A better understanding of host-pathogen interaction within the nasal vestibulum and the nasal mucosa is therefore crucial to develop new strategies to diminish the risk of invasive *S. aureus* infection.

Antimicrobial peptides (AMPs) or cationic host defense peptides are indispensable parts of innate immunity [3]. AMPs are

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evolutionarily conserved effector molecules that exhibit a broad spectrum of antimicrobial activity against Gram-positive and -negative bacteria, fungi and also against some encapsulated viruses [4]. AMPs are expressed along the internal and external barriers of multicellular organisms i.e. the skin or mucous membranes and are present in specific secretory cells or leukocytes [5]. AMPs are either constitutively expressed or induced upon inflammatory stimuli or bacterial contact [6].

Within the last decade progress has been made in the understanding of the impact of AMPs in innate immunity. This holds particularly true for AMPs as an integral part of the cutaneous innate immune defense against invading pathogens. In keratinocytes AMPs are induced by inflammatory cytokines such as IL-1α, IL-1β, IFN-γ and TNF-α [5,7,8]. Moreover, recent evidence suggests a role of Th17 cytokines in induction of RNase7 and β-defensins in primary keratinocytes [9–12]. The synergistic action of Th17 as well as inflammatory cytokines was shown to be crucial for AMP induction in keratinocytes and bronchial epithelial cells but not for other cell types – an observation that may explain the susceptibility of patients with hyper IgE-syndrome (that are deficient in Th17 cytokines) to *S. aureus* infections of the skin and the lung [13].

Beside inflammatory cytokines pathogen-associated molecular patterns (PAMPs) induce AMPs via pattern recognition receptors (PRRs) like Toll-like receptors (TLRs). In support of this, recent studies have shown that bacterial culture supernatant (BCM) [14], viable and heat-inactivated *S. aureus* as well as lipoteichoic acid (LTA) [15] induced AMP expression in primary keratinocytes.

Accumulating evidence suggests that keratinocyte-derived AMPs play a role in *S. aureus* colonization and infection of the skin. The human AMP RNase7 helps to prevent *S. aureus* colonization or to control *S. aureus* growth on the skin [16,17]. Accordingly, individuals with deficient hBD3 induction after sterile wounding of healthy skin in vivo were found to be prone to permanent *S. aureus* nasal carriage [18] and more severe *S. aureus* skin infection [19].

However, as the human nares/the nasal vestibulum represents the primary niche in which *S. aureus* resides, investigations of the host-pathogen interplay should not only include the skin i.e. keratinocytes and cutaneous appendages but also elucidate innate defense mechanisms in the nasal mucosa. We therefore focused our work on AMP expression in primary nasal epithelial cells (HNEC) and the interaction of these cells with *S. aureus*. Aims of the current study were a) to investigate the impact of inflammatory cytokines and Th17 cytokines on AMP expression in HNEC, b) to analyze if a differential AMP induction pattern can be observed in HNEC by colonizing and invasive *S. aureus* as well as commensal bacteria and c) whether the human nasal septum squamous cell carcinoma cell line, RPMI2650, may be used as a model to investigate AMP expression patterns in nasal mucosa. We thus investigated the expression of hBD3, RNase7 and LL-37 in HNEC, RPMI2650 and HaCat (as control cell line) *in vitro* upon stimulation with a classical (IFN-γ, IL-1β, TNF-α) and a Th17 cytokine cocktail (IL-17A, IL-17F, IL-22). Additionally the AMP inducibility by BCM of different colonizing and invasive *S. aureus* isolates as well as *Staphylococcus epidermidis* isolates was studied.

2. Materials and methods

2.1. Cell culture

The spontaneously immortalized cell line HaCat [20] was kindly provided by Prof. Dr. Karin Loser (University Hospital Muenster, Germany). HaCats were cultured in RPMI 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Sigma Aldrich, Seelze, Germany), sodium pyruvate (Sigma Aldrich, Seelze, Germany) 1 mM Hepes (Life Technologies, Darmstadt, Germany) and non-essential amino acids (Biochrom, Berlin, Germany).

RPMI2650, human nasal septal squamous cell carcinoma cell line [21] was maintained in Earle's minimum essential medium (MEM) (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, non-essential amino acids, sodium pyruvate and 2 mM L-glutamine (Life Technologies, Darmstadt, Germany).

Primary human nasal epithelial cells (HNEC) (PromoCell, Heidelberg, Germany) were cultured in Airway Epithelial Cell Growth Medium (PromoCell, Heidelberg, Germany). HNEC were used from passage 2 for 14 population doublings.

For stimulation experiments HaCat, RPMI2650 and HNEC were seeded in T25 cell culture flasks or 6-well cell culture plates and were grown until 80–90% confluence. Two hours before stimulation both cell lines (HaCat, RPMI2650) were washed once with PBS (Life Technologies, Darmstadt, Germany) or HNEC with HBSS (Promocell Heidelberg, Germany), then medium was replaced by serum and antibiotic free culture medium (RPMI2650). HaCats were stimulated in keratinocyte-serum free medium (Life Technologies, Darmstadt, Germany) and HNEC were stimulated in Airway Epithelial Cell Basal Medium (Promocell Heidelberg, Germany). Two hours later, this medium was replaced by serum and antibiotic free culture medium (RPMI2650, HNEC) or by keratinocyte-serum free medium (KSF-M) (HaCats) containing cytokines or bacterial preparations.

2.2. Bacteria and preparation of bacterial conditioned medium

Bacteria used in the study are outlined in Table 1. Overnight cultures of *S. aureus*, or *S. epidermidis* isolates were prepared in Tryptic Soy Broth with shaking, 100 rpm at 37 °C. Bacteria were adjusted to 1×10^8 CFU/ml and 1×10^9 CFU/ml by addition of respective amounts of KSF-M (HaCat), serum or antibiotic free culture medium (RPMI2650, HNEC). Bacteria-containing medium was then centrifuged for 5 min at 4000 rpm, supernatant was sterile filtered and cells were stimulated with the resulting BCM for 24 h. Cells incubated with fresh serum or antibiotic free culture medium supplemented with the same concentration of TSB served as a negative control.

2.3. Stimulation with cytokines and with LTA

Cells were stimulated with the classical cytokines IL-1β (10 ng/ml), TNF-α (10 ng/ml) or IFN-γ (10 ng/ml) (PeproTech, Hamburg) or a combination of these in the same concentrations (designated as classical cytokine cocktail). The Th17 cytokine cocktail consisted of IL-17F (200 ng/ml), IL-17A (20 ng/ml) and IL-22 (200 ng/ml) (eBioscience, United Kingdom and PeproTech, Hamburg). When the different cells were stimulated with a combination of both cytokine cocktails the same concentrations as in the individual cytokine cocktails were used. Cells were stimulated for 12, 24 and 48 h (HaCat and RPMI2650) or for 24 and 48 h (HNEC).

HaCat and RPMI2650 cells were treated with 10 μg/ml LTA (Sigma Aldrich, Seelze, Germany) for 6, 12 and 24 h. HNEC were treated with 10 μg/ml LTA (Sigma Aldrich, Seelze, Germany) for 24 h.

2.4. Real time polymerase chain reaction analysis (RT-PCR)

Total RNA was isolated from cell pellets with the RNeasy Mini Plus Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Before RNA isolation Qiashredder columns (Qiagen) were used in order to homogenize the cells. The iScript cDNA-synthesis