

Defective killing of *Staphylococcus aureus* in atopic dermatitis is associated with reduced mobilization of human β -defensin-3

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Background: Individuals with atopic dermatitis (AD) have frequent colonization and infection with *Staphylococcus aureus*. Rapid elimination of *S aureus* depends on constitutive synthesis and mobilization of human β -defensin-3 (HBD-3).

Objective: To determine whether keratinocytes in AD, compared with normal, skin are less able to kill *S aureus* rapidly, and to assess the potential role that abnormally low mobilization of HBD-3 onto *S aureus* has in this process.

Methods: Skin samples from 10 normal individuals and 10 patients with AD were compared for synthesis and mobilization of HBD-3 onto surface-associated *S aureus*. Furthermore, keratinocytes from 10 individuals were studied for the effects of T_H2 cytokines on the ability of the cells to synthesize and mobilize HBD-3, and to kill *S aureus*.

Results: Keratinocytes in skin biopsies from subjects with AD were defective in killing *S aureus* relative to normal individuals ($P < .001$). The constitutive levels of HBD-3 in the epidermal keratinocytes were similar between normal individuals and those with AD. However, the cells of patients with AD were unable to mobilize HBD-3 efficiently to kill *S aureus*. Physiologic Ca⁺⁺ was essential for development of normal HBD-3 levels by cultured human keratinocytes. Mobilization of HBD-3 and the ability to kill *S aureus* were significantly ($P < .05$) inhibited by IL-4 and IL-13. Antagonism of IL-4/10/13 with antibodies significantly ($P < .01$) improved mobilization of HBD-3 onto the surface of *S aureus* by skin from patients with AD.

Conclusion: Patients with AD have problems with *S aureus* skin infection. This is a result of increased levels of T_H2 cytokines, which inhibit keratinocyte mobilization of HBD-3. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Antimicrobial peptides, defensins, human, bacterial infections, skin, keratinocytes

Abbreviations used

AD: Atopic dermatitis
DAPI: 4'-6-Diamidino-2-phenylindole, dihydrochloride
HBD-3: Human β -defensin-3
KGM: Keratinocyte Growth Medium
MRSA: Methicillin-resistant *Staphylococcus aureus*

The skin is the primary interface between the human body and the environment. As such, it is continually exposed to microbes and forms an important barrier preventing the invasion of microbes into the body. Atopic dermatitis (AD) is a highly pruritic, chronic inflammatory skin disease that affects as many as 20% of children worldwide and can persist into adulthood.^{1,2} It has a significant impact on the quality of life of patients and their families; patients with AD have frequent bacterial and viral skin infections.³⁻⁵ Even normal-appearing skin of patients with AD is heavily colonized by *Staphylococcus aureus*.³ Methicillin-resistant *S aureus* (MRSA) has emerged as a worldwide pathogen that has rapidly evolved from a cause of nosocomial to community-acquired infections in recent years. Patients with AD are susceptible to colonization and infection by MRSA because they are frequently treated, often for extended courses, with antistaphylococcal antibiotics.⁶ Thus, patients with AD may serve as an important vector for transmission of these bacteria in the community. Understanding the underlying mechanisms for infection and colonization by *S aureus* of the skin of patients with AD is critical in devising more effective treatment strategies for this serious public health issue.

The epidermis is a dynamic structure, with the keratinocytes in a state of constant growth and differentiation.⁷ As the keratinocytes move from the basal to the apical layers and differentiate, they experience increasing concentrations of extracellular calcium.⁸ During differentiation, expression of a number of different genes are upregulated and downregulated, consistent with the functions of the keratinocytes in particular layers of the epidermis.⁹ This mode of regulation extends to cytokine receptors and host defense peptides.¹⁰

It has been established clearly that the overabundance of T_H2 cytokines in the skin of patients with AD can severely attenuate the upregulation of antimicrobial responses in response to bacterial and viral stimuli.^{11,12} Therefore, attenuation of the antimicrobial responses within the skin of patients with AD may allow microbes to persist once they are deposited in AD lesions. However, this does not explain the higher burden of bacteria in the cornified layers, and among the epidermal keratinocytes in nonlesional skin of patients with AD. We have recently reported that human β -defensin-3

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(HBD-3) is the key constitutively expressed molecule that keratinocytes in normal human skin use to kill *S aureus* rapidly and that this mechanism for *S aureus* killing is acquired by human epidermal keratinocytes as they pass through the extracellular calcium differential from the basal to the apical layers of the epidermis.¹³ This mechanism was dependent on constitutive synthesis of HBD-3, accumulation of the peptide in the cytoplasm, and then mobilization of the cytoplasmic stores onto surface-bound *S aureus*. In the current study, we therefore examined whether atopic skin was defective in *S aureus* killing and the role of reduced HBD-3 synthesis and/or mobilization in this process.

METHODS

Skin biopsies

Subjects included 10 patients with AD diagnosed according to the Hanifin and Rajka¹⁴ criteria, and 10 healthy individuals with no history of skin disease. None of the patients had previously received oral corticosteroids or cyclosporin, and topical corticosteroids were not allowed for more than 1 week before enrollment. This study was approved by the institutional review board at National Jewish Medical and Research Center in Denver, and all patients gave written informed consent before participation in these studies. Four 2-mm skin biopsies were collected from each donor and cultured overnight at 37°C, 5% CO₂, to allow for involution of damaged cells at the cut edge of the tissue. The biopsies were then exposed to *S aureus* (Wood-46) labeled with Texas Red for 5 to 60 minutes, after which they were rinsed 3 times with Keratinocyte Growth Medium (KGM, Cambrex, Inc, East Rutherford, NJ) and fixed with 4% paraformaldehyde.

Keratinocytes

Normal human neonatal keratinocytes were obtained from Cambrex (Walkersville, Md) and used as previously described.¹³

S aureus

S aureus ATCC strain numbers 29213 and 10832 (Wood-46) were used in our experiments. *S aureus* was grown overnight in Tryptic Soy Broth (Becton Dickinson, Franklin Lakes, NJ) at 37°C and labeled as previously described.¹³ Bacterial concentration was determined by OD reading of the bacteria measured in a spectrophotometer.

Bactericidal assay

Twenty-four hours before the experiment, the cell culture medium was removed and the cultures rinsed 3 times with 37°C KGM. The medium was replaced with antibiotic-free KGM containing all supplements and 1.3 mmol/L CaCl₂ for controls. Different wells were also supplemented with 50 ng/mL IL-4 and IL-13. At the time of the experiment, fresh antibiotic-free medium with 1×10^6 bacteria/mL was inoculated into each well. Cells were coincubated with bacteria. Lysed, viable, and total bacteria were counted as previously described.¹³

Imaging

The Wood strain, ATCC 10832, was used for imaging, because of markedly reduced nonspecific antibody binding.¹⁵ The bacteria were surface labeled with 10 μ mol/L Texas Red, succinimidyl ester as previously described.¹³ Keratinocytes were cultivated and differentiated as described in 24-well plates containing 12-mm round #1 cover glasses (Fisher Scientific, Pittsburgh, Pa). The fluorescently labeled bacteria were then added to the keratinocyte cultures for 1 hour, after which the unbound bacteria were removed by vigorous rinsing and the cultures fixed with 4% paraformaldehyde overnight at 4°C.

For staining with antibodies to HBD-3, samples were permeabilized and blocked with PBS containing 3% BSA, 5% human serum, and 0.1% Tween 20 (staining buffer). Polyclonal rabbit anti-HBD-3 at 10 μ g/mL (1:100; Origen, San Diego, Calif) or preimmune serum was diluted as noted and previously described.¹³

Calculation of contact-mediated *S aureus* destruction in skin

Texas Red fluorescence of the intact bacteria was quantitated by first identifying intact bacteria in contact with the keratinocytes. Bacteria were scored as intact for this analysis if they presented a 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI)-positive nucleus surrounded by a Texas Red-positive cell envelope. The cumulative Texas Red fluorescence associated with those structures was taken as the numerator for each tissue sample, and at each edge of the epidermal sheet. The denominator was taken as the total Texas Red fluorescence, after subtraction of the background fluorescence, in the edges of the epidermal layers. The percentage of destroyed *S aureus* was calculated as $1 - (\text{fluorescence associated with intact } S \text{ aureus} / \text{total Texas Red fluorescence in the edge of the epidermal sheet}) \times 100$.

Quantitation of mRNA

Total RNA was isolated from normal skin explants by chloroform:phenol extraction and isopropanol precipitation according to the manufacturer's guidelines (Sigma Chemical Co, St Louis, Mo). RNeasy Mini Kits (Qiagen, Valencia, Calif) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to purify RNA further from skin explants as previously described.¹³ Real-time PCR was performed and analyzed by the dual-labeled fluorogenic probe method using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, Calif) as previously described.¹³

Quantitation of HBD-3 exposure in cultured keratinocytes

Synthetic HBD-3 was the generous gift of Wuyuan Lu at the University of Maryland. Quantitation of antimicrobial peptide exposure of the bacteria was conducted as previously described.^{13,16} The lyophilized peptide was resuspended at 1 mg/mL in 0.01% acetic acid, aliquoted, and stored at -80°C until use. Polyclonal rabbit anti-HBD-3 at 10 μ g/mL was obtained from Origen and was used diluted to 1:100. Intensity values of at least 50 bacteria per concentration were used to construct standard intensity versus exposure curves for HBD-3. The intensity values after subtraction of the average background of samples exposed to control antibody were fit to lines by using regression analysis. The line was considered a valid representation of the data if a correlation coefficient (R^2) of 0.95 or greater was reported. Keratinocyte samples stained and imaged in the same experiment as the standard samples were imaged by using identical image capture settings as the standards. At least 20 bacteria in each of triplicate wells were imaged, and each bacterium's intensity was interpolated to equivalent *in vitro* HBD-3 exposure via the line equation. Equivalent exposure values were then compared with samples stained with the appropriate control antibody via *t* test.

Quantitation of HBD-3 exposure in human skin was analyzed by using similar techniques, with the exception that 4 biopsies were examined from each of 4 individuals. Serial sections from each biopsy were stained and imaged until at least 10 bacteria in contact with the keratinocyte layers were observed for each biopsy, for a total of 40 bacteria per antimicrobial peptide stain per individual. Intensities of each bacterium were interpolated to equivalent antimicrobial peptide exposure via the standard curves and the values compared with bacteria in serial sections stained with control antibody. Average equivalent *in vitro* exposure values for each stain were compared with control antibody via *t* test, and a *P* value less than .05 was considered significant.

Statistical analyses

S aureus killing assays were analyzed by comparing the means of total versus viable bacteria from triplicate samples receiving different treatments using the Student *t* test. Mann-Whitney analysis was used to compare means of data that were not normally distributed, such as *S aureus* destruction by human skin. PCR ratios of target mRNA to 18s RNA in specific donor responses were compared via *t* tests for paired samples. *P* values less than .05 were considered significant. Each comparison was performed 3 times.

RESULTS

Epidermal keratinocytes in skin of patients with AD are defective in destruction of *S aureus*

In studies examining the interaction of fluorescently labeled *S aureus* with the epidermal keratinocytes in biopsies of normal skin, we noted that the bacterial structures rapidly became disorganized, the nuclei disappeared, and the cell envelopes became condensed. With increasing time, the covalently attached fluorescent label that was initially associated with the *S aureus* became diffuse and distributed throughout the cytoplasm of the keratinocytes.¹³ After 1 hour of exposure of *S aureus* to the biopsies from normal individuals in the current study, only 1.2% of the bacteria remained intact (mean $\pm$ SD = 1.2 ± 1.19 ; Fig 1, B). Most of the fluorescence initially associated with the bacteria became distributed throughout the keratinocytes (Fig 1, C), and the bacterial nuclei were no longer detectable via DAPI staining (Fig 1, B). In contrast, the epidermal keratinocytes in samples of skin from patients with AD showed 16.3% *S aureus* remaining morphologically intact after 1 hour (mean $\pm$ SD = 16.3 ± 22.8), and less of the fluorescence was distributed away from the bacteria and distributed within the cytoplasm of the keratinocytes (Fig 1, A). This novel defect in the ability of skin from patients with AD to destroy *S aureus* rapidly was highly significant (Fig 1, C; $P < .001$, Mann-Whitney). Therefore, the reason that patients with AD have a higher burden of *S aureus* on their skin can be directly attributed to a defect in contact-mediated killing by the epidermal keratinocytes.

Reduced HBD-3 mobilization in AD keratinocytes

We have previously shown that rapid contact-mediated killing of *S aureus* by keratinocytes is dependent on the synthesis and mobilization of HBD-3 by the epidermal keratinocytes in normal individuals.¹³ Therefore, we investigated the ability of keratinocytes from patients with AD, compared with normal subjects, to synthesize and mobilize HBD-3. In these experiments, biopsies taken from normal donors or from uninvolved skin of patients with AD were exposed to fluorescently labeled *S aureus*. After fixation and staining for HBD-3, we determined the relative levels of HBD-3 staining in the samples and the amount of HBD-3 mobilized onto the bacteria bound to the epidermal keratinocytes of the skin. Fig 2, A and B, are representative images from 1 normal and 1 AD skin sample after 1 hour of exposure to *S aureus*. Analysis of biopsies (4 each) from 10 patients with AD and 10 normal donors showed that the mean fluorescence intensity of HBD-3 staining in the normal epidermal keratinocytes was 6.9 ± 1.035 and $7.4 \pm .41$ (SEM) in the AD keratinocytes. This difference was not statistically significant ($P > .05$, t test; Fig 2, C).

We next analyzed the HBD-3 intensity on the surface-bound *S aureus* and compared it with a standard curve of HBD-3 binding versus concentration in both AD and normal skin (Fig 2, D). This revealed that although *S aureus* bound to keratinocytes in normal skin was exposed to approximately 34.3 ± 2.4 (SEM; range, 22.2–45.7 $\mu\text{mol/L}$) HBD-3, bacteria bound to keratinocytes in uninvolved AD skin were exposed to only 12.2 ± 3.0 (SEM; range, 4.4–29.1 $\mu\text{mol/L}$) HBD-3. Because the minimum bactericidal concentration of HBD-3 for *S aureus* is approximately 10.7 $\mu\text{mol/L}$, many of the bacteria in the AD skin were not exposed to enough HBD-3 to kill them.

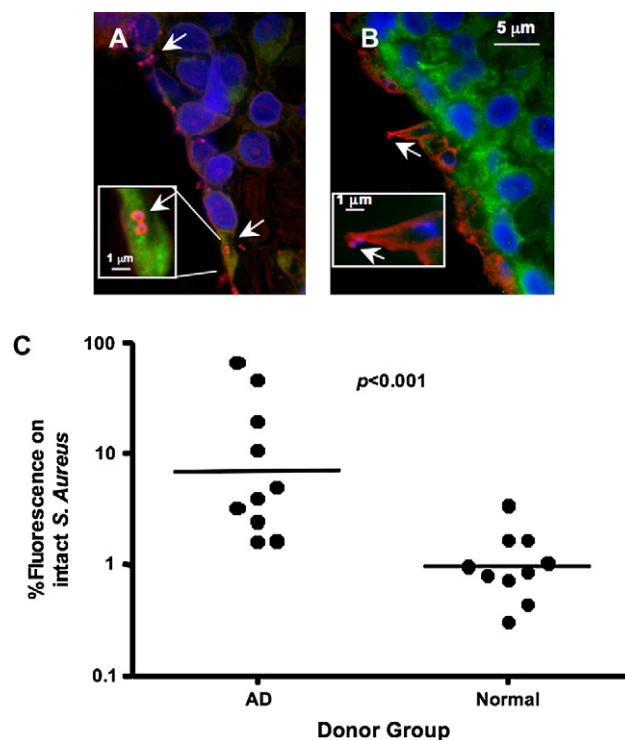


FIG 1. Impaired destruction of *S aureus* by epidermal keratinocytes in AD skin. Biopsies were taken from 10 normal individuals and 10 patients with AD. They were imaged after 1 hour of incubation with Texas-Red–labeled *S aureus*. **A**, Epidermal keratinocytes at the edge of the skin sample from a representative patient with AD, showing much intact *S aureus* (arrow). Scale bar represents 5 μm (inset scale bar = 1 μm). **B**, Epidermal keratinocytes at the edge of the skin sample from a representative normal individual, showing DAPI-stained nuclei (blue), and the remains of *S aureus* (red). **C**, Quantitation of samples from 10 normal subjects and 10 patients with AD for the percentage of red fluorescence associated with intact *S aureus*. Means were compared via Mann-Whitney test ($P < .001$).

IL-4 and IL-13 inhibit killing of *S aureus* by keratinocytes

IL-4 and IL-13 are increased in the unaffected skin of patients with AD.^{17,18} We therefore investigated whether IL-4 and IL-13 inhibited the ability of cultured human keratinocytes to rapidly kill surface-bound *S aureus*. The results of these experiments showed that the percentage of bound *S aureus* that was killed after contact with the keratinocytes decreased by an average of 2-fold in cultures exposed to IL-4 and IL-13 for 24 hours ($P < .05$, t test for paired samples; Fig 3, A). Examination of keratinocytes from 10 different donors showed a range of inhibition of killing from 0% to 65%.

We and others have previously observed that the induction of antimicrobial peptides, including defensins, by proinflammatory stimuli in the skin of patients with AD is inhibited by IL-4 and IL-13.^{12,19} Given the 2 observations that several defensins and LL37 are present in the keratinocytes of normal skin,¹³ and that induction of differentiation with Ca^{++} results in expression of HBD-3 and other antimicrobial peptides, we asked whether the high levels of IL-4 and IL-13 present in AD skin²⁰ could interfere with the differentiation-associated, constitutive synthesis of HBD-3. We examined this by culturing keratinocytes for 5 to 7 days in the presence of KGM alone or KGM containing 1.3 mmol/L Ca^{++} . During the final 24 hours, cultures were incubated

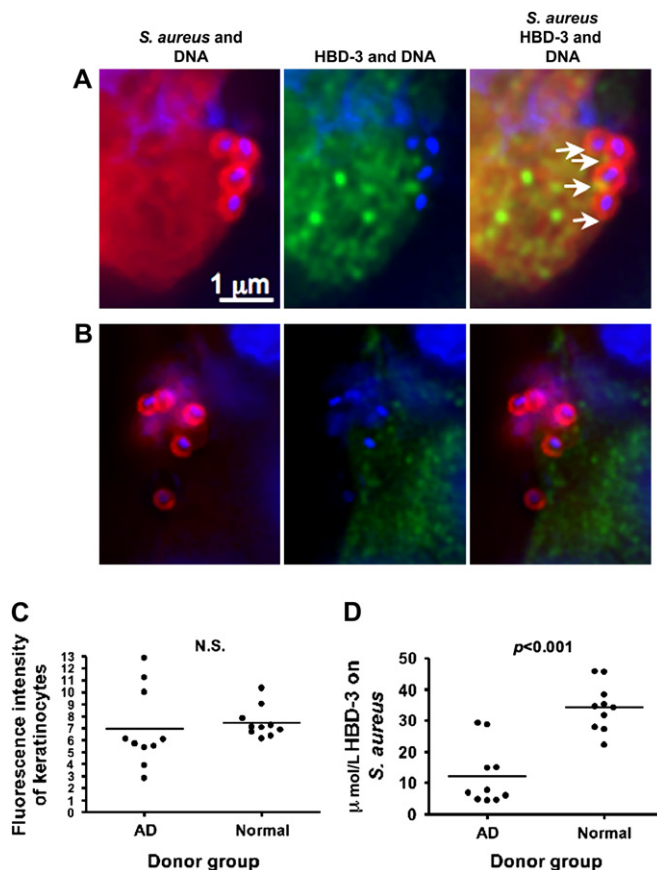


FIG 2. Reduced HBD-3 mobilization in AD skin. Biopsies from 10 normal subjects and 10 patients with AD were exposed to *S aureus* (red) and then counterstained for HBD-3 (green). Nuclei are shown in blue. The left panels show the red (*S aureus* cell envelope) and blue (DNA) channels, the center panels show the green (HBD-3) and blue (DNA) channels, and the right panels show the merged images. **A**, Representative image of the epidermis of normal skin. Rare examples of *S aureus* have been included, showing swelling of the bacteria and apparent disorganization of the cell envelope during destruction. **B**, Representative image of epidermis of AD skin. Scale bar represents 1 μ m. Arrows indicate areas of high colocalization of HBD-3 with the *S aureus* cell envelope. **C**, Quantitation of relative fluorescence (green) after staining AD and normal skin for HBD-3. **D**, Deposition of HBD-3 on cell-associated *S aureus* interpolated from a standard curve of HBD-3 binding versus concentration. Significance of mean differences was analyzed via Mann-Whitney. N.S., Not significant.

in the presence and absence of IL-4 and IL-13. Fig 3, B, shows that relative to KGM alone, the presence of 1.3 mmol/L Ca^{++} caused the keratinocytes to increase the levels of mRNA encoding HBD-3 by the cells of all 10 donors ($P < .05$, t test). However, the presence of IL-4 and IL-13 in the cultures for the final 24 hours had no significant effect on HBD-3 mRNA expression ($P > .05$, t test). Therefore, although Fig 3, A, shows that IL-4 and IL-13 strongly inhibit keratinocyte killing of surface bound *S aureus*, the effect did not appear to be caused by inhibition of HBD-3 expression.

IL-4 and IL-13 inhibit mobilization of HBD-3 by keratinocytes

This experiment was performed with keratinocytes from each donor, and the results are shown in Fig 4, A and B. Because IL-4 and IL-13 had no effect on the HBD-3 mRNA levels in the cells

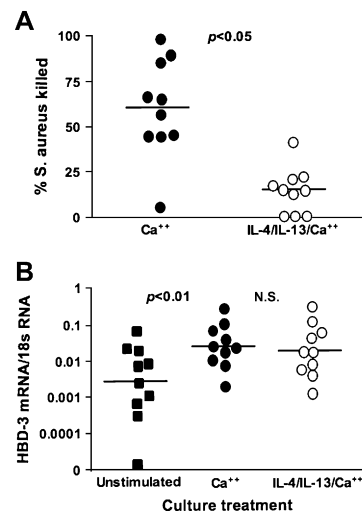


FIG 3. Effects of $\text{T}_\text{H}2$ cytokines on constitutive killing of *S aureus* by human keratinocytes, and expression of HBD-3 mRNA. **A**, *S aureus* was exposed to keratinocytes from 10 different subjects. The average percentage of bound *S aureus* killed in the presence or absence of IL-4 and IL-13 is shown for each donor. $P < .05$, t test for paired samples. **B**, Geometric mean expression of HBD-3 mRNA in the presence of 0.06 mmol/L Ca^{++} (Control), 1.3 mmol/L Ca^{++} (Ca^{++}), and 1.3 mmol/L Ca^{++} with IL-4 and IL-13 (IL4/IL-13/ Ca^{++}) as measured by quantitative PCR in keratinocytes from 10 donors. Means were compared via t test for paired samples. N.S., Not significant.

(Fig 3, B), we also examined the amount of peptide present in the cytoplasm in the cells. These results showed that there was no significant difference in HBD-3 peptide levels as a result of IL-4 and IL-13 treatment (Fig 4, A). However, when we examined the cells for the ability to mobilize HBD-3 from the cytoplasm onto the surface of *S aureus*, we found that IL-4 and IL-13 significantly inhibited mobilization of this antimicrobial peptide (Fig 4, B; $P < .05$, t test for paired samples). Therefore, the effect of IL-4 and IL-13 in cultured keratinocytes is mainly to inhibit mobilization of HBD-3 rather than synthesis or accumulation of the peptide in the cytoplasm.

Antagonism of IL-4, IL-13 and IL-10 in AD skin enhances deposition on *S aureus*

To examine directly whether $\text{T}_\text{H}2$ cytokines inhibit the ability of keratinocytes to kill *S aureus* rapidly in the epidermis by interfering with synthesis and/or mobilization of HBD-3, we incubated biopsies of skin from patients with AD in medium containing neutralizing antibodies to $\text{T}_\text{H}2$ cytokines (IL-4, IL-10, and IL-13) as we have done in previous studies.¹⁹ After 24 hours of exposure to the antibodies, we exposed AD skin to *S aureus* and examined the mobilization of HBD-3 onto *S aureus* bound to the epidermal keratinocytes. The results indicated that relative to control antibodies, antibodies to IL-4, IL-13, and IL-10 did not cause a significant increase in the amount of HBD-3 expressed in the skin samples (Fig 5, A; $P > .05$, t test for paired samples). However, the antibodies caused a 3-fold ($P < .01$, t test for paired samples) increase in the amount of HBD-3 deposited onto the surface of *S aureus* (Fig 5, B). Therefore, these $\text{T}_\text{H}2$ cytokines inhibited the ability of keratinocytes to mobilize HBD-3 from the cytoplasm onto the surface of bacteria in the skin of patients with AD. Importantly, antagonism of $\text{T}_\text{H}2$ cytokines in skin from patients with AD was able to enhance mobilization of HBD-3 independently of any effect on synthesis of HBD-3. Taken

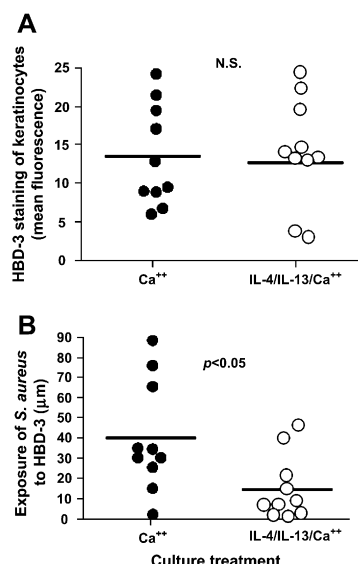


FIG 4. Effect of T_H2 cytokines on constitutive synthesis and mobilization of HBD-3. Keratinocytes from 10 donors were differentiated in the presence of 1.3 mmol/L Ca^{++} for 7 days. For the last 24 hours, the cultures were incubated in medium only, or medium supplemented with IL-4 and IL-13. **A**, Staining intensity for HBD-3 in the keratinocytes, expressed in mean fluorescence units. **B**, Mobilization of HBD-3 onto *S aureus* bound to keratinocytes of 10 donors in the presence and absence of IL-4 and IL-13. Groups were compared via *t* test for paired samples. N.S., Not significant.

together, these data highlight the process of peptide mobilization as a key step in the ability of epidermal keratinocytes to kill microbes in the skin. This is a defect that has not been well described in previous reports examining the skin innate immune response in human disease.

DISCUSSION

The skin of individuals with AD is frequently colonized and infected by *S aureus*, whereas these bacteria are rarely found on the skin of normal subjects.^{21,22} In the current study, we have demonstrated directly that the epidermal keratinocytes in samples of AD skin are deficient in their ability to destroy *S aureus* that comes into contact with them. It is known that antimicrobial peptides play an important role in controlling colonization and growth of microbes at epithelial surfaces,^{23,24} including the skin.¹³ This previous work primarily focused on the induction of antimicrobial peptides 24 hours after inflammatory stimulation or injury to the epithelium. Recently we reported that HBD-3 is a key player in the constitutive ability of differentiating keratinocytes from normal subjects to kill *S aureus* rapidly (within 1 hour) in the absence of inflammation. It was therefore of interest to determine whether there may be differences in the ability of keratinocytes from patients with AD to synthesize and/or use HBD-3 to kill *S aureus*.

For keratinocytes to use HBD-3 in the constitutive killing mechanism, they must generate cytoplasmic stores of this peptide, and they must be able to mobilize it to bind to the bacteria at the cell surface. Mobilization of antimicrobial effector molecules from the cytoplasm is often associated with mast cells, macrophages, and neutrophils. These antimicrobial effector cells use cell surface receptors to initiate signal transduction cascades involving calcium entry into the cytosol²⁵⁻²⁷ and release of

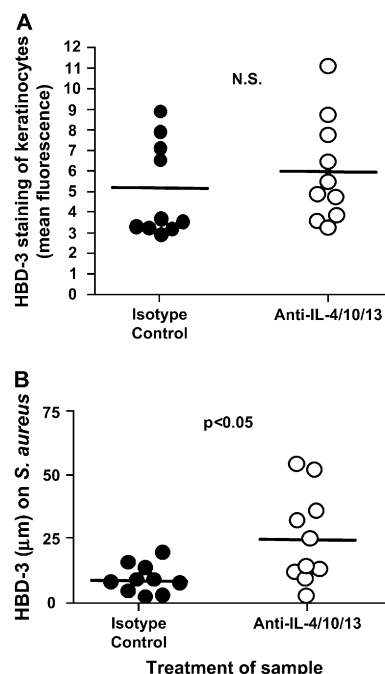


FIG 5. Response of skin of patients with AD to antagonism of T_H2 cytokines. Four biopsies of nonlesional skin were harvested from each of 10 patients with AD. Biopsies from each donor were cultured overnight with isotype control in KGM, or in KGM-containing antibodies to IL-4, IL-13, and IL-10. **A**, Overall content of HBD-3 in the skin biopsies. **B**, Amount of HBD-3 secreted onto surface-bound *S aureus*. Means were compared by *t* test for paired samples. N.S., Not significant.

internal stores from the endoplasmic reticulum. The sudden rise in cytosolic Ca^{++} concentration, or spike, triggers multiple membrane-membrane fusion events that can result in exocytosis, fusion of phagosomes with vesicles, or degranulation.

Although there was no significant difference in constitutive HBD-3 levels in the skin of patients with AD relative to normal individuals, we observed that subjects with AD were unable to mobilize and deposit normal amounts of HBD-3 onto *S aureus*. The epidermal keratinocytes in AD skin were not able to deposit sufficient amounts of HBD-3 onto the surface of *S aureus* to kill all of the *S aureus* efficiently.¹³ In the same experiment, the epidermal keratinocytes in normal skin deposited sufficient HBD-3 onto the bacteria to kill greater than 99.9% of them, based on the sensitivity of these bacteria to HBD-3 in culture. This was not an artifact of different numbers of *S aureus* in contact with the keratinocytes, because our previous work has shown that the system is tolerant of wide ranges of bacteria without significantly depleting cellular stores of HBD-3.¹³ Therefore, keratinocytes from patients with AD had a decreased ability to mobilize this peptide onto the bacteria at the cell surface. Although there have been numerous studies on the effects of T_H2 cytokines on inducible expression of antimicrobial peptides,^{12,28} there have been no previous studies of their effects on mobilization of antimicrobial peptides by epithelial cells.

One important difference between patients with AD and normal individuals is that patients with AD have higher levels of T_H2 cytokines, both systemically and in the skin.^{12,22} We and others have previously observed that the T_H2 cytokines IL-4 and IL-13 strongly inhibit expression of antimicrobial peptides by keratinocytes in response to inflammatory stimuli, such as

bacterial products and T_H1 cytokines.¹¹ This is likely a key element supporting large bacterial burdens in AD skin lesions.³ In this study, we hypothesized that T_H2 cytokines might play a similar, inhibitory role on the microbicidal capacity of differentiating keratinocytes outside of lesions. However, in contrast with the inhibitory efforts of the T_H2 cytokine on induced HBD-3, IL-4 and IL-13 did not have an effect on constitutive synthesis of HBD-3. Nevertheless, incubation of keratinocyte cultures with IL-4 and IL-13 for 24 hours before the addition of *S aureus* showed that the microbicidal activity against *S aureus* was significantly inhibited (Fig 3). This is consistent with the observation in Fig 2, D, that in AD skin, there is less HBD-3 deposition onto bacterial surfaces. Therefore, this supports the hypothesis that the reason there are more bacteria on and in the unaffected skin of patients with AD is that the high levels of IL-4 and IL-13 in patients with AD inhibit the constitutive, rapid killing mechanism for *S aureus* that exists in normal skin.¹³

Our observation that IL-4 and IL-13 did not affect the constitutive expression of HBD-3 suggested IL-4 and IL-13 must be able to disrupt the killing at another step distinct from HBD-3 synthesis. In this study, we make the novel observation that this involved mobilization of HBD-3 from the cytoplasm onto the bacterial surface. Identification that mobilization of HBD-3 can be independently inhibited by T_H2 cytokines in keratinocytes represents an additional novel finding of our study.

To support the physiologic role of T_H2 cytokines in the impairment of *S aureus* killing that we observed in AD skin, we incubated skin samples from patients with AD with antibodies to the T_H2 cytokines, IL-4, IL-10, and IL-13, for 24 hours before addition of *S aureus* to the samples. The rationale for this experiment was to try to neutralize the inhibitory activity of T_H2 cytokines remaining in the sample after collection, or newly secreted by cells within the skin samples.¹⁹ The treatment of the skin samples with antibodies once again had no effect on the amount of HBD-3 in the epidermal keratinocytes. However, we noted that deposition of HBD-3 to the surface of *S aureus* was significantly enhanced ($P < .05$; Fig 5, B). Therefore, it appears that T_H2 cytokines can interfere with the mobilization of cytoplasmic HBD-3 onto surface-bound *S aureus* independently of their effects on synthesis in human skin samples as well as in cultured keratinocytes.¹⁹

In summary, patients with AD are uniquely susceptible to colonization and infection by pathogens such as *S aureus* on the skin. Because AD is the most common chronic inflammatory skin disease and such patients can be a vector for transmission of MRSA, this is a serious global health issue with implications beyond dermatologic management of AD. We observed that epidermal keratinocytes from patients with AD have reduced ability to destroy *S aureus* that comes in contact with their keratinocytes, because they could not mobilize microbicidal levels of HBD-3 onto the surface of *S aureus*. Additional studies showed that inhibition of mobilization could be reproduced in cultured keratinocytes by incubation with the T_H2 cytokines IL-4 and IL-13 and that antagonism of IL-4/IL-10/IL-13 in AD skin reversed the inhibition of mobilization. Therefore, we conclude that patients with AD bear abnormally high burdens of *S aureus* on the skin because high levels of T_H2 cytokines interfere with constitutive killing by epidermal keratinocytes. This is consistent with previous reports that anti-inflammatory agents, such as topical steroids and calcineurin inhibitors, can reduce *S aureus* colonization.^{29,30} The

mechanism of this interference lies in inhibition of HBD-3 mobilization from the keratinocyte cytoplasm, rather than inhibition of HBD-3 synthesis. Strategies to reverse this interference should include new therapeutic approaches based on immunomodulation of the innate immune response. This approach may be preferable to use of antibiotic therapy, which can contribute to resistant organisms.

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Key messages

- Epidermal keratinocytes of people with AD lack the rapid, constitutive mobilization of HBD-3 onto *S aureus* relative to normal skin.
- Development of rapid, constitutive bactericidal activity is dependent on Ca^{++} and differentiation. Bactericidal activity is strongly inhibited by T_H2 cytokines via inhibition of HBD-3 mobilization.
- Mobilization of bactericidal concentrations of HBD-3 in AD skin can be enhanced by inhibition of IL-4, IL-10, and IL-13 activity.

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