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Antimicrobial Activity of Murine Lung Cells against *Staphylococcus aureus* Is Increased In Vitro and In Vivo after Elafin Gene Transfer

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*Staphylococcus aureus* is a pathogen often found in pneumonia and sepsis. In the context of the resistance of this organism to conventional antibiotics, an understanding of the regulation of natural endogenous antimicrobial molecules is of paramount importance. Previous studies have shown that both human and mouse airways express a variety of these molecules, including defensins, cathelicidins, and the four-disulfide core protein secretory leukocyte protease inhibitor. We demonstrate here by culturing mouse tracheal epithelial cells at an air-liquid interface that, despite the production of Defb1, Defb14, and Defr1 in this system, these cells are unable to clear *S. aureus* when exposed to this respiratory pathogen. Using an adenovirus (Ad)-mediated gene transfer strategy, we show that overexpression of elafin, an anti-elastase/antimicrobial molecule (also a member of the four-disulfide core protein family), dramatically improves the clearance of *S. aureus*. In addition, we also demonstrate that this overexpression is efficient in vivo and that intratracheal instillation of Ad-elafin significantly reduced the lung bacterial load and demonstrates concomitant anti-inflammatory activity by reducing neutrophil numbers and markers of lung inflammation, such as bronchoalveolar lavage levels of tumor necrosis factor and myeloperoxidase.

These findings show that an increased antimicrobial activity phenotype is provided by the elafin molecule and have implications for its use in *S. aureus*-associated local and systemic infections.

*Staphylococcus aureus* infections are closely associated with pneumonia and sepsis, particularly in nosocomial infections (12, 13). This organism can often cause primary pneumonias, in young adults and patients with cystic fibrosis (5, 8, 9). Although pulmonary infections with *S. aureus* could until recently be controlled with aggressive antibiotic therapy, the increasing association of *S. aureus* with antimicrobial resistance has become a major concern for clinicians.

In that context, endogenous antimicrobial molecules (AMMs) are gathering interest as alternative microbicidal agents since they are thought to be less likely to induce bacterial resistance than conventional antibiotics (10). This class of molecules includes defensins, cathelicidins, and the four-disulfide core proteins secretory leukocyte protease inhibitor and elafin (29–31). In parallel with human airways, respiratory tract epithelia of mice (a useful species in which to model lung infections) have been shown to express β-defensin molecules, including Defb1 (18), Defb2 (19), Defb3 (2), Defb4 (14), Defb6 (38), Defr1 (21), and the cathelicidin CRAMP (24).

Although little is known about the in vivo activity of these molecules against *S. aureus*, we have shown that chemically synthesized β-defensin Defb1 and Defr1 murine peptides have antimicrobial activity in vitro against this microorganism (18, 21). However, deletion of Defb1 by gene targeting did not lead to increased airway retention of this pathogen after exposure in vivo (20, 22). This suggests that the peptide is either redundant or not crucial in innate immunity against *S. aureus* in the airways. To investigate innate defenses against *S. aureus* at the mucosal surface of the airways and to facilitate screening of other potentially important AMMs, we used primary cultures of differentiated mouse tracheal epithelia (at an air-liquid interface [ALI]) as previously described (4). We demonstrate here that Defb1, Defb14, and Defr1 (the orthologue of human β-defensin 3, *DEFB103*), which display potent activity against *S. aureus* in vitro (17), are expressed in these epithelial cell cultures. In addition, we show that despite expressing a repertoire of peptides capable of killing *S. aureus* in vitro, primary ALI cultured cells are unable to clear this respiratory pathogen. In contrast, we show there that adenovirus (Ad)-mediated overexpression of human elafin/skin-derived antileucoprotease/trappin-2 (16, 31, 34, 37), a 9.9-kDa neutrophil elastase inhibitor with antimicrobial activity against *S. aureus* and *Pseudomonas aeruginosa* (when added as a purified molecule, leads to killing of *S. aureus* both in vitro and in vivo in a murine acute lung infection model. This demonstrates that elafin overexpression may have potential therapeutic benefit against *S. aureus* infections.

**MATERIALS AND METHODS**

Recombinant replication-deficient Ad constructs. Two E1-partially E3-deleted type 5 recombinant replication-deficient adenoviruses were used. The Ad-LacZ...
construct was a gift from J. Gaudie and F. Graham (1), whereas the Ad-elafin construct was generated as described elsewhere [28].

**Bacteria.** *S. aureus* C1705 (a clinical strain [9, 18]) was grown initially as colonies on Columbia agar (Unipath, Basingstoke, United Kingdom) and then in 10 ml of tryptone soy broth (Unipath) overnight at 37°C in an orbital shaker (Gallenkamp; Fisher Scientific, Loughborough, United Kingdom) at 200 rpm at room temperature. The resulting suspension was centrifuged at 3,000 rpm for 5 min at room temperature. The supernatant was discarded, and the pellet resuspended in 10 ml of phosphate buffer (8 mM K2HPO4, 2 mM KH2PO4). Suspensions were adjusted with phosphate buffer to an A260 of 1.50, yielding an estimated bacterial concentration of 10^8 CFU/ml, in accordance with predetermined growth curves.

Serial dilutions were performed with phosphate buffer to obtain the desired concentration of viable bacteria (see below).

**Mouse epithelial cell culture.** Female C57BL/6 or BALB/c murine primary tracheal epithelial cells were generated and grown on semipermeable membranes at an ALI according to our previously published method (4). Briefly, tracheae were excised and severed at the proximal limit of the thyroid cartilage and at the junction of the main bronchi. The thyroid gland and other adherent tissues were removed before cutting tracheally longitudinally. Dissociated cells from two tracheae (ca. 4 × 10^8 cells) were seeded onto one semipermeable support membrane, precoated with 0.5 mg of collagen/ml (24-well plate inserts, mated bacterial concentration of 10^9 CFU/ml, in accordance with predetermined growth curves.) After 18 to 20 h at 37°C (see above), 20 µl of either phosphate buffer or a C1705 *S. aureus* suspension (10^9 CFU/ml) was added to the cells with the use of a special Hamilton precision syringe (Hamilton Company, Reno, NV), followed by incubation for 3 h at 37°C. Cell inserts were washed with both 10 µl of phosphate buffer and 10 µl of phosphate buffer-0.5% Triton X-100. These two washes were then pooled and centrifuged at 8,000 rpm for 10 min. Supernatants were used for elafin quantitation by enzyme-linked immunosorbent assay (ELISA) [27], whereas bacterial cell pellets were suspended in PBS and used for bacterial counts on Columbia agar. Agar plates were incubated overnight at 37°C prior to colony counting. Values represent the mean ± the standard error of three experiments, each performed in triplicate. (Significant differences [P < 0.05] compared to Ad-lacZ-infected cells are indicated in Table 1.)

**In vivo studies.** Female C57BL/6 mice between 6 and 8 weeks old (Harlan Olac, Bicester, United Kingdom) were treated intratracheally (as described in reference 33) with 40 µl of Ad-LacZ (adenovirus control) or Ad-elafin (3 × 10^7 PFU in both cases). After 5 days, 4 × 10^7 CFU of *S. aureus* (in 40 µl) were administered via the same route. This bacterial dose was shown in previous pilot studies to produce a local inflammatory response in the lung. At 24 h after the bacterial administration, mice were euthanized and bronchoalveolar lavage (BAL) performed (two serial instillations of 0.4 ml of PBS). BAL fluid (BALF) was centrifuged 10 min at 4,000 × g; supernatants were stored at −20°C until further use, whereas each cell pellet was resuspended in 100 µl of PBS. Cell counting (with a hemocytometer) and cytospins (300 rpm for 3 min at room temperature) were then performed. After BALF was recovered, the lungs were harvested for either RNA preparation (storage in RNA later [Sigma]), histology, or bacterial counts. For the latter, half lungs were homogenized in 2 ml of PBS by using a tissue homogenizer (OMNI µH International; Camlab), and homogenates were further diluted 10-fold with PBS. A total of 100 µl of the diluent was plated on Columbia agar plates, which were incubated at 37°C for 24 h.

**RESULTS**

Murine defensin expression in murine primary epithelial cells. RNA was prepared from C57BL/6 tracheal inserts as detailed in Materials and Methods, and RT-PCR was performed with primers for *Hprt* (house keeping gene), as well as for the three murine β-defensin genes relevant to antimicrobial activity against *S. aureus*. Figure 1 demonstrates that murine C57BL/6 tracheal cells express Defb1, the mouse orthologue of human *DEFB1*, and Defb14, the mouse orthologue of *DEFB103*. We also show for the first time that Defb1 is expressed in murine epithelial cells. There is no evidence for significant upregulation of these genes after LPS exposure.

**Innate antimicrobial activity of murine epithelial cells against *S. aureus*.** We were interested in examining whether any difference in bacterial killing was evident between BALB/c and 3610 M CMICHAEL ET AL. INFECT. IMMUN.
and C57BL/6 mice since they have a polymorphism in one of their β-defensin genes. BALB/c has Defb8, a classic six cysteine β-defensin, whereas C57BL/6 has a polymorphic allele with three nucleotide and amino acid changes that results in a five-cysteine β-defensin termed Defr1. This molecule has a novel structure, which when made synthetically has potent antibacterial activity (3). Despite the expression of β-defensins in C57BL/6 tracheal cells, neither these cells nor cells derived from BALB/c mice were able to inhibit the growth of an approximately 200 CFU S. aureus inoculum (20 nl of a 10⁷-CFU/ml S. aureus suspension) (Fig. 2). This was considered already as a very small inoculum (identical to that used by Smith et al. [36]), and no attempt was made to further reduce that dose. Indeed, at 3 h postinoculation, an average of 3,077 and 2,050 CFU were recovered from C57BL/6 and BALB/c cells, respectively, compared to 329 in the original inoculum. This clearly demonstrates that, far from being bacteriostatic or bactericidal, the tracheal epithelium was able to promote S. aureus growth under these conditions.

With this observation in mind, we examined whether recom-
binant adenovirus (Ad)-mediated overexpression of human elafin, an antimicrobial molecule with no ortholog in mice, was able to limit \textit{S. aureus} growth in vitro and in vivo.

**Optimization of infection of murine C57BL/6 epithelial cells with Ad vectors.** We first tested the Ad expression system by using the \textit{E. coli} β-galactosidase reporter gene (Ad-LacZ). Because others have reported poor adenoviral gene transfer to airway epithelia due to low apical expression of the coxsackie/adenovirus receptor (26), we used a method incorporating the use of CaPO$_4$ precipitates in the infection protocol in the present study, as previously investigated (6).

Indeed, we show here (Fig. 3) that generation of CaPO$_4$ precipitates in MEEM (a medium particularly well suited for the generation of the latter due to its high concentration of PO$_4$ ions) containing Ad-LacZ significantly increased (two- to threefold) the efficiency of infection of murine C57BL/6 tracheal cells compared to Ad-LacZ infection in the USG medium usually used for these cells (Fig. 3, compare panels D and C). This amended protocol, with MEEM as the medium of choice, was therefore used for all subsequent experiments. Neither CaPO$_4$- nor Ad-mediated cytotoxicity was detected by trypan blue exclusion (not shown).

**In vitro antibacterial activity of Ad-elafin against \textit{S. aureus}**. Having established the conditions for optimal Ad infection of murine C57BL/6 tracheal cells, we infected these cells with Ad-elafin and used Ad-LacZ as an Ad control. Figure 4 shows that, in accordance with all our previous studies (28, 30, 32), murine cells do not produce an elafin-related protein since our ELISA did not detect a cross-reacting species from untreated cells or cells treated with Ad-LacZ. In contrast, Ad-elafin infection induced the expression and secretion of very high levels of human elafin (right panel). This level correlated with an efficient reduction of \textit{S. aureus} numbers (left panel, \( P < 0.005 \)).

**In vivo antibacterial activity of Ad-elafin against \textit{S. aureus}**. When studying antimicrobial activity against \textit{P. aeruginosa} (33), we have shown that Ad-LacZ was a useful Ad vector control which, by itself, did not induce a detectable increase in lung inflammation in comparison to PBS treatment. This lack of overt inflammation caused by Ad-LacZ is most probably due to the very low dose of Ad used in our study (3 \( \times 10^7 \) PFU). In

![Image](image1.jpg)

**FIG. 3.** Optimization of infection of murine C57BL/6 tracheal cells with Ad-LacZ. Female C57BL/6 mouse tracheal epithelial cells cultured on inserts (see above) were infected with Ad-LacZ at an MOI of 100, in the presence or absence of CaPO$_4$ precipitates (final concentration of 5.8 mM Ca$^{2+}$ and 0.86 mM PO$_4$) in MEEM or USG medium. Cells were incubated with this infection medium in a total volume of 250 \( \mu \)l for 45 min at 37°C. Cells were then washed twice with PBS and incubated for 18 to 20 h at 37°C in the absence of media bathing the apical surface. (A) No Ad, USG medium; (B) no Ad, MEEM plus CaPO$_4$; (C) Ad-LacZ, USG medium; (D) Ad-LacZ, MEEM plus CaPO$_4$. 

![Image](image2.jpg)
the present study we compared two experimental groups, Ad-LacZ (3 \times 10^7 PFU) plus *S. aureus* and Ad-elafin (3 \times 10^7 PFU) plus *S. aureus*, and one control group, PBS-PBS; the latter served as a control for potential background *S. aureus* commensals and contamination in the laboratory.

As expected and in keeping with our previous studies (and present in vitro data), no elafin mRNA was detected by RT-PCR in mice receiving Ad-LacZ or PBS, demonstrating that the band detected in the Ad-elafin–*S. aureus* group (Fig. 5, upper right) was specific to the human elafin transgene. This elafin expression was associated with a reduction in the number of recovered *S. aureus* in the BALF of Ad-elafin–treated mice, compared to mice given Ad-LacZ (Fig. 5, left panel; *P* = 0.038). As expected, no bacteria were recovered in the mice treated twice with PBS.

**Modulation of in vivo inflammatory responses by Ad-elafin gene transfer.** When inflammatory cell profiles in BALF were studied, compared to the PBS/PBS group, unsurprisingly, both the Ad-LacZ plus *S. aureus* and the Ad-elafin plus *S. aureus* groups showed increased numbers of total inflammatory cells, particularly neutrophils (*P* = 0.02 and 0.013, respectively). However, no statistically significant differences in cellular profiles were observed between the Ad-LaZ plus *S. aureus* and Ad-elafin plus *S. aureus* groups (data not shown). Interestingly, when the Ad-elafin plus *S. aureus* group was considered in isolation, there was a strong negative correlation between BAL elafin levels and neutrophil numbers (Fig. 6, *r* = −0.63, *P* = 0.069), suggesting that elafin may be associated in this model with a reduction in neutrophil numbers. Also, MPO levels, used as a marker of neutrophil activation, were lower in the Ad-elafin plus *S. aureus* group, although the difference did not reach statistical significance. A similar reduction (statistically significant) in the proinflammatory mediator TNF-α was noted in the Ad-elafin–*S. aureus* group, compared to the Ad-LacZ–*S. aureus* group (Table 1). When total protein levels were measured as an index of blood and/or alveolar barrier disruption, levels were lower in the PBS/PBS group compared to the Ad-groups, but within these there was a trend toward reduction of protein levels in the Ad-elafin–*S. aureus* group. Mirroring these data, blinded histological analysis showed neutrophilic inflammatory foci in the Ad-LacZ–treated animals, which were reduced in the Ad-elafin–treated mice. Quantification of neutrophils in whole lung sections is also given (representative sections are shown in Fig. 7).

**DISCUSSION**

*S. aureus* is a major human pathogen associated with localized and systemic infections. In order to facilitate and model the study of microbial infections in mice, cell culture systems have been developed where epithelial cells form a confluent, polarized, ciliated epithelium when grown at an air-liquid interface (4). Using this model, we have previously demonstrated that murine tracheal epithelial cells display many characteristics similar to those of murine tracheal epithelium in vivo and that these cells retain the ability ex vivo to produce important antimicrobial molecules such as β-defensins, which have been specifically demonstrated in the trachea (4). We have also shown previously that *Defb2* is expressed in these cells after LPS induction (4). However, we did not follow the in vivo fate of this gene in relation to *S. aureus* exposure because, in vitro, the *Defb2* peptide is a very poor antimicrobial against *S. aureus*.
strain C1705 (M. Rolfe et al., unpublished). Here, we have extended these findings by showing for the first time that two further important molecules with activity against *S. aureus* (*Defr1* and *Defb14*) are also expressed in this system. *Defr1* encodes a defensin related peptide (Defr1) that deviates from the canonical six cysteine defensin motif by having the first cysteine replaced with a tyrosine in C57BL/6 mice (21). Surprisingly, we have shown that the five cysteine Defr1 peptide is active in vitro as an antimicrobial and has salt sensitive activity against *S. aureus*.

*Defb14* is the mouse orthologue of human *DEFB103*, which encodes a human peptide (hBD3), shown to have potent activity against *S. aureus*, including MRSA (17). However, despite the ability of these cells to secrete these antibacterial molecules (Fig. 1), we have shown here that they are unable to control *S. aureus* growth in ALI cultures (Fig. 2).

The mechanism for such resistance of *S. aureus* may be complex and could be due partly to its capacity to modify phosphatidylglycerol, the major phospholipid of *S. aureus*, with L-lysine, rendering the membrane more cationic and hence repulsive to cationic antimicrobial molecules such as defensins (25). A more specific mechanism could be the production of staphylokinase, which has been shown recently to inactivate human defensins (15).

Although we have not demonstrated here the secretion of murine endogenous antimicrobial peptides (and only showed their transcripts [see Fig. 1]), it is likely that these were also produced as demonstrated for HBD-2 in the same ALI system (35). Since it is therefore likely that *S. aureus* was able to grow even in the presence of these endogenous peptides, we then tested whether overexpressing human...
elafin, a molecule for which no ortholog exists in the mouse, would endow murine tracheal cells with defense against \textit{S. aureus}.

Here, using the Ad-overexpressing system described above, we show that murine tracheal epithelial cells producing human elafin were able to reduce \textit{S. aureus} numbers significantly (Fig. 4). Although the exact mechanism of action was not elucidated and was beyond the scope of the present study, the elafin molecule has a net positive charge of +7, suggesting that its activity against \textit{S. aureus} (34) may be directed at bacterial membranes.

We show in Fig. 4 that elafin secretion is associated with significant inhibition of growth of \textit{S. aureus}, although it is unclear whether it acts only to slow the growth of the organisms, directly kills a proportion of the organisms, or a combination of both, since the CFU counts of \textit{S. aureus} recovered after incubation with Ad-elafin-infected cells were not lower than those added at the outset (not shown). In addition, elafin

\begin{table}
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\begin{tabular}{|l|c|c|c|}
\hline
Treatment & No. of mice & BALF protein (\text{\mu g/ml}) & BALF MPO (arbitrary units) & BALF TNF (pg/ml) \\
\hline
PBS-PBS & 3 & 314.7 (232.7–320.4) & 0.031 (0.008–0.26) & 15.0 (3.75–48.75) \\
Ad-LacZ--\textit{S. aureus} & 6 & 743.2 (579.3–851.6) & 0.094 (0.049–0.299) & 2,283.3* (975.0–3,225.0) \\
Ad-elafin--\textit{S. aureus} & 8 & 567.0* (433.7–689.3) & 0.059 (0.027–0.165) & 577.5† (221.9–1,188.1) \\
\hline
\end{tabular}
\caption{Inflammatory parameters in BALF of C57BL/6 mice sequentially receiving Ad vectors and \textit{S. aureus}.}
\end{table}

* Female C57BL/6 mice received sequential intratracheal treatments of Ad-LacZ or Ad-elafin and then \textit{S. aureus} as described in Materials and Methods. At 24 h after bacterial administration, BALF was obtained and centrifuged; the cell pellet was used for differential cell analysis, whereas supernatants were used for the determination of total protein and TNF levels and MPO activity (given as median values). *, significantly different from the PBS-PBS group; †, significantly different from the Ad-LacZ--\textit{S. aureus} group (Mann-Whitney, \(P < 0.05\)).

\textbf{FIG. 7.} Ad-elafin treatment results in the reduction of neutrophil clusters after intratracheal administration of \textit{S. aureus} in mice. The histological appearance (magnification, \(\times 400\)) of representative 3-\textmu m lung sections (fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin) is shown. (b and c) Ad-elafin--\textit{S. aureus} treatment reduced the numbers of neutrophil clusters (occasional “isolated” neutrophils were observed, see arrows) compared to Ad-LacZ--\textit{S. aureus} treatment, where multiple clusters were present (b, arrows). (a) None were seen in PBS-PBS-treated animals. Corresponding neutrophil numbers from each section are shown in the relevant bar graph.
S. aureus

tion factor which has been shown to be activated by inhibits NF-
eutrophil-associated "collateral lung damage," as shown by a reduction in the cellular inflammatory response, as exemplified reducing the infective load. A consequence of this would be a reduced in the Ad-elafin–plus–bacteria group. It could there-

taneous, when the Ad-elafin group was considered on its own, there was a negative correlation between lung elafin levels and neutrophil numbers (Fig. 6). The histological analysis of lungs confirmed this finding and showed a reduction in the number of neutrophilic pneumonia-like foci in the alveoli (Fig. 7). This is reminiscent of our previous study, in which P. aeruginosa was administered intratracheally in a protocol similar to the one used here (33). In that study, as here (Table 1), markers of inflammation such as BALF TNF-α and MPO levels were reduced in the Ad-elafin–plus–bacteria group. It could there-fore be hypothesized that, in an infective situation, elafin may act directly as a bacteriostatic or bactericidal agent, thereby reducing the infective load. A consequence of this would be a reduction in the cellular inflammatory response, as exemplified by the reduction in cytokine levels, neutrophil activation, and neutrophil-related "collateral lung damage," as shown by a reduction in protein leakage in the alveolar compartment (Table 1). Elafin could also have a further direct anti-inflammatory function; indeed, we have shown recently that in vitro elafin inhibits NF-κB (11), an important proinflammatory transcription factor which has been shown to be activated by S. aureus protein A (7). Of note, the mode of action of elafin may be different in noninfective situations, for example, when LPS is used as an inflammatory stimulus, where elafin’s chemotactic functions may become prominent (30, 32).

In conclusion, we demonstrated here that elafin is able to confer an increased antimicrobial phenotype to primary murine tracheal epithelial cells which, despite the endogenous expression of at least three β-defensins (shown at the RNA level) with in vitro relevance to S. aureus killing, were otherwise unable to contain the growth of this pathogen in vitro.

This phenotype was replicated in vivo, in a model of acute inflammation in C57BL/6 mice. We believe that these data have implications both for the development of improved mu-rine models of S. aureus lung infections and for the consider-ation of elafin as a potential therapeutic agent against S. au-reus-associated local and systemic infections.

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