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(2011) Journal of Innate Immunity, 3 (5), pp. 495-507.

Research Article

Journal of Innate Immunity

J Innate Immun 2011;3:495–507
DOI: 10.1159/000327714

Received: February 8, 2011
Accepted after revision: March 22, 2011
Published online: June 8, 2011

Staphylococcus aureus Evades the Extracellular Antimicrobial Activity of Mast Cells by Promoting Its Own Uptake

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Key Words
Mast cells · *Staphylococcus aureus* · Antimicrobial activity · Intracellular persistence

Abstract
In this study, we investigated the interactions of *Staphylococcus aureus* with mast cells, which are multifunctional sentinels lining the surfaces of the body. We found that bone marrow-derived murine mast cells (BMMC) exerted a powerful phagocytosis-independent antimicrobial activity against *S. aureus*. Both the release of extracellular traps as well as discharge of antimicrobial compounds were the mechanisms used by the BMMC to kill extracellular *S. aureus*. This was accompanied by the secretion of mediators such as TNF-α involved in the recruitment of effector cells. Interestingly, *S. aureus* subverted the extracellular antimicrobial activity of the BMMC by internalizing within these cells. *S. aureus* was also capable to internalize within human mast cells (HMC-1) and within murine skin mast cells during in vivo infection. Bacteria internalization was, at least in part, mediated by the α5β1 integrins expressed on the surface of the mast cell. In the intracellular milieu, the bacterium survived and persisted by increasing the cell wall thickness and by gaining access into the mast cell cytosol. The expression of α-hemolysin was essential for staphylococci intracellular persistence. By hiding within the long-life mast cells, staphylococci not only avoid clearance but also establish an infection reservoir that could contribute to chronic carriage.

Introduction

Staphylococcus aureus is a major human pathogen that can cause a broad spectrum of serious community-acquired and nosocomial infections including skin infections, bacteremia, pneumonia and sepsis [1]. The treatment of *S. aureus* infections has been complicated by the widespread emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* [2]. The substantial challenge posed by methicillin-resistant *S. aureus* makes it imperative to develop more efficient therapeutic strategies. For this reason, a better understanding of the natu-

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ral antimicrobial mechanisms mobilized by the host immune defenses to defeat *S. aureus* will facilitate the design of such therapeutic options.

S. aureus is generally a commensal organism that persistently colonizes the anterior nares of 20% of the human population [3]. Invasive staphylococcal infections are usually the result of a breach in the epithelial barriers that allows the pathogen to gain access to deeper tissue [4]. During this process, *S. aureus* will be initially confronted by the host innate immune defense. However, the mechanism by which the innate immune response to *S. aureus* is initiated is not completely understood. While polymorph nuclear neutrophils have been implicated in the elimination of *S. aureus*, it is conceivable that other immune cells may also participate in the vigorous host response to staphylococcal invasion.

Mast cells are key elements of the innate immune surveillance present at sites that are constantly exposed to the environment and where pathogens are frequently encountered [5]. Mast cells are very well known for their major role in allergic reactions mediated by their high-affinity receptors for IgE [6]. However, in addition to their role in allergy, numerous studies have reported that mast cells have an important function in host defense against pathogens [7–9]. Thus, mast cells can recognize infecting pathogens and respond to infection by secreting inflammatory mediators such as TNF-α and chemokines required for the rapid recruitment of effector cells [10]. Furthermore, several studies have indicated that mast cells can also exert a direct antimicrobial activity against pathogens by diverse mechanisms including opsonin-mediated phagocytosis and killing [11], secretion of compounds that are toxic for pathogens [12] or by releasing extracellular structures composed of DNA, histones and mast cell granule proteins that entrap and kill bacteria [13, 14]. Overall, it is now clear that an important effector function of mast cells is to promote innate immunity against microbial pathogens.

In this study, we have performed an in-depth characterization of the interactions between *S. aureus* and mast cells, including primary bone marrow-derived murine mast cells (BMMC) and the human mast cell line HMC-1. Our results show that mast cells exerted a phagocytosis-independent antimicrobial activity against *S. aureus* that was mediated by both the release of extracellular traps as well as by the secretion of antimicrobial compounds. We also found that *S. aureus* subverted this extracellular antimicrobial activity by internalizing within the mast cell. Intracellular *S. aureus* survives in the intracellular milieu by gaining access into the mast cell cytosol and by reprogramming gene transcription including the upregulation of cell wall synthesis. In summary, the mast cell can act as a double-edged sword during *S. aureus* infection. On the one hand, mast cell activation during the early innate immune response may help to limit the infection. On the other hand, by providing shelter to *S. aureus,* they can also act as a long-term bacterial reservoir supporting bacterial persistence and chronic carriage.

Materials and Methods

Bacterial Strains

The *S. aureus* strains used in this study were strain SH1000 and its α-hemolysin-deficient ($hla^{-/-}$) isogenic mutant strain [15], as well as the green fluorescent protein (GFP)-expressing SH1000 [16]. Staphylococci were grown to the mid-log phase at 37°C with shaking (150 rpm) in brain heart infusion (BHI) medium, collected by centrifugation, washed with sterile PBS, and diluted to the required concentration. The number of viable bacteria was determined after serial diluting and plating on BHI-agar.

For some experiments *S. aureus* was heat killed by incubating the organisms at 90°C for 60 min. The killed cultures were subcultured on blood agar plates at 37°C for 24 h to prove that no viable organisms remained.

Generation of BMMC

BMMC were isolated from 4- to 6-week-old female C57BL/6 mice (Winkelmann, Borchen, Germany). Briefly, mice were sacrificed by CO_2 asphyxiation, their femurs and tibias removed, and bone marrow cells were harvested by repeated flushing with Iscove's modified Dulbecco's medium (IMDM). Bone marrow cells were incubated in IMDM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 20 U/ml of recombinant murine IL-3 for 21 days. Nonadherent cells were transferred to fresh culture plates every 2–3 days to remove adherent macrophages and fibroblasts. The purity of the resulting cell population consisted of >98% of mast cells as determined by flow cytometry analysis using anti-mouse CD117 antibody (Caltag Laboratories, Hamburg, Germany) and a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, Calif., USA).

Human Mast Cell Line HMC-1 Culture Conditions

The human mast cell line HMC-1 [17] used in this study was a generous gift from Dr. J. Butterfield (Mayo Clinic, Rochester, Minn., USA). HMC-1 cells were maintained in IMDM supplemented with 10% FCS, 1.2 mM α-thioglycerol, 100 U/ml penicillin and 100 μg/ml streptomycin.

BMMC and Staphylococci Co-Cultivation Assay

BMMC were harvested, washed twice with IMDM without antibiotics and seeded in 48-well tissue culture plates at a density of 2×10^6 cells/ml. *S. aureus* was added to the wells containing BMMC at a multiplicity of infection (MOI) of 1 bacterium per BMMC (1:1) or to wells containing medium alone. Kinetics of bacterial growth in the presence or absence of BMMC was monitored at increasing times of infection by determination of colony-forming units (CFU) in the culture supernatants.

In some experiments, BMMC were treated for 30 min prior to infection with either 10 μg/ml cytochalasin D (Sigma, Deisenhofen, Germany), 1 μM of the NADPH oxidase inhibitor diphenylene iodonium (Sigma), 1 μM Ca^{2+} ionophore ionomycin (Sigma), 100 μM cromolyn (Sigma) or 50 mU of micrococcal nuclease (New England Biolabs, Frankfurt am Main, Germany).

Visualization of Mast Cell Extracellular Traps

To examine the release of extracellular traps by fluorescence microscopy, BMMC were seeded on poly-L-lysine-covered glass cover slips, infected with *S. aureus* at a MOI of 1:1 and fixed with 4% paraformaldehyde at 4 h of infection. BMMC were then stained using the LIVE/DEAD cell viability kit for mammalian cells (Invitrogen, Karlsruhe, Germany) following the manufacturer's recommendations and examined using a Zeiss Axiophot microscope with an attached Zeiss Axiocam HRc digital camera and Axiovision software 4.7 (Carl Zeiss, Oberkochen, Germany).

LIVE/DEAD BacLight™ Bacterial viability kit (Invitrogen) was used in some experiments to determine the viability of *S. aureus* following the recommendations of the manufacturer.

Degranulation Assays

Mast cells were stimulated either by aggregation of the high-affinity IgE receptor (FcεRI) by using murine IgE (USBio, Hamburg, Germany) and anti-IgE antibodies (Abcam, Cambridge, UK) in 1 ml IMDM medium, supplemented with 5% FCS, for 2 h at room temperature or by exposing them to *S. aureus*. Mast cells were centrifuged at progressing times after stimulation, and degranulation was determined by assessing the percentage of β-hexosaminidase and tryptase released into the culture supernatant. A 40-μl volume of culture supernatant and 100 μl of 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidase in 0.04 M sodium citrate buffer (pH 4.5) were added to each well of a 96-well plate, and color was allowed to develop for 30 min at 37°C. The enzyme reaction was terminated by adding 200 μl 0.2 M glycine-NaOH (pH 10.7). The absorbance at 405 nm of each sample was measured with a 96-well TECAN Sunrise reader (Tecan Group Ltd., Männedorf, Switzerland). The cell pellets were lysed with distilled H_2O and the extracts were analyzed for the total β-hexosaminidase activities, which in untreated mast cells (spontaneous release) were subtracted from the enzyme activity (test sample release). The percentage of β-hexosaminidase released into the supernatant was calculated using the following formula: release (%) = (test – spontaneous)/(total – spontaneous) × 100.

The release of tryptase was quantified by using the mast cell degranulation assay kit (Millipore, Schwalbach/Ts., Germany), according to the manufacturer's instructions. In brief, each sample was incubated in a substrate solution (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in 0.04 M sodium citrate, pH 4.5) at 37°C for 60 min. The reaction was stopped by the addition of 0.2 M glycine (pH 10.0). The absorbance at 405 nm of each sample was measured with a 96-well TECAN Sunrise reader (Tecan Group Ltd.).

The levels of TNF-α were determined by using the BD OptEIA™ mouse TNF-ELISA Set (mono/poly) (BD Biosciences, San Diego, Calif., USA) according to the manufacturer's instructions.

Gentamicin/Lysostaphin Protection Assay

Mast cells were resuspended in DMEM medium with 10% FCS in 48-well plates at 1 × 10^6 cell/ml, infected with *S. aureus* at a MOI of 20:1 and incubated for 2 h at 37°C. Infected mast cells were washed three times with PBS to remove non-adherent bacteria and remained extracellular bacteria were eliminated by treatment with 20 μg/ml of lysostaphin for 30 min. Mast cells were then extensively washed and either used for the visualization or quantification of internalized *S. aureus* or resuspended in fresh medium containing 100 μg/ml gentamicin and further incubated for various periods of time. To enumerate intracellular bacteria, infected mast cells were harvested, washed with PBS and the cellular pellet lysed with 0.1% Triton X-100 in PBS. Recovered intracellular bacteria were quantified by plating serial dilutions on BHI agar plates and enumerating colony counts.

In some experiments, BMMC were pre-incubated with anti-β1 integrin antibodies (New England Biolabs) for 2 h at 37°C.

Immunofluorescence Microscopy

For double immunofluorescence of extracellular/intracellular bacteria, mast cells were applied to sterile cover slips and exposed to *S. aureus* (MOI 20:1) for 2 h. Cover slips were then rinsed to remove unbound cells, and adherent cells were fixed with 4% formaldehyde. For double immunofluorescence staining, extracellular bacteria were stained with polyclonal rabbit anti-*S. aureus* antibodies, followed by Alexa green-conjugated goat anti-rabbit antibodies (Sigma). After several washes, cells were permeabilized by 0.025% Triton X-100 in PBS, washed again, and intracellular bacteria were stained by anti-*S. aureus* antibodies, followed by Alexa red-conjugated goat anti-rabbit antibodies (Sigma). The percentage of mast cells with intracellular *S. aureus* (red fluorescence) was determined with an Axiophot microscope (Zeiss).

Field Emission Scanning Electron Microscopy

HMC-1 were fixed with 4% paraformaldehyde, washed with TE buffer (20 mM TRIS, 1 mM EDTA, pH 6.9), dehydrated by incubating with a graded series of ethanol (10, 30, 50, 70, 90, 100%) on ice for 15 min, critical-point dried with liquid CO_2 (CPD 30; Balzers Union, Balzers, Liechtenstein), and covered with a gold film by sputter coating (SCD 40; Balzers Union) before being examined in a field emission scanning electron microscope (Zeiss DSM 982 Gemini, Carl Zeiss, Jena, Germany) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

Transmission Electron Microscopy

Mast cells were fixed with 2% glutaraldehyde and 3% formaldehyde in cacodylate buffer for 1 h on ice, washed with cacodylate buffer, and osmificated with 1% aqueous osmium for 1 h at room temperature. Samples were then dehydrated with a graded series of acetone (10, 30, 50, 70, 90 and 100%) for 30 min at each step. Dehydration in the 70% acetone step was done with 2% uranyl acetate overnight. Samples were infiltrated with an epoxy resin according to the Spurr formula [18]. Ultrathin sections were cut with a diamond knife, counterstained with uranyl acetate and lead citrate, and examined in a TEM910 transmission electron microscope (Carl Zeiss) at an acceleration voltage of 80 kV. Images were taken at calibrated magnifications using a line replica. Images were recorded digitally with a Slow-Scan CCD-Camera (1,024 × 1,024; ProScan, Scheuring, Germany) with ITEM Software (Olympus Soft Imaging Solutions, Münster, Germany). Brightness and contrast were adjusted with Adobe Photoshop CS3.

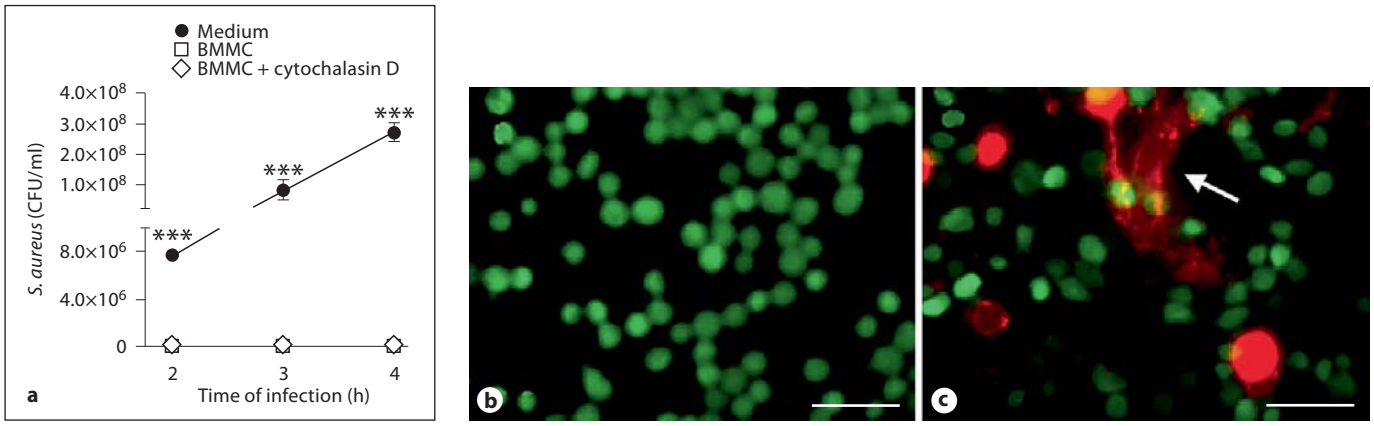

Fig. 1. Extracellular killing of *S. aureus* by BMMC. **a** Growth of *S. aureus* in medium alone, in co-culture with BMMC or in co-culture with cytochalasin D-treated BMMC. Data are expressed as CFU of *S. aureus* per milliliter of culture supernatant. Each point represents the mean ± SD of the compilation of triplicates from 3 independent experiments. *** $p < 0.001$ for *S. aureus* growth in medium alone versus *S. aureus* growth in the presence of untreated or cytochalasin D-treated BMMC. **b**, **c** Immunofluorescence photograph showing BMMC releasing extracellular traps at 4 h of co-culture with *S. aureus* (**c**, white arrow). Uninfected BMMC are shown in **b**. Staining of BMMC was performed using the LIVE/DEAD cell viability kit for mammalian cells. Dying cells releasing DNA appear in red and viable cells in green. ×88. Scale bars = 30 μm.

Skin Infection Model

Female BALB/c mice (8–10 weeks old) purchased from Harlan-Winkelmann (Borchen, Germany) were used for experimental infection. Mice were anesthetized with Isofluran (Isoba®; Essex Tierarznei, München, Germany) and infected subcutaneously with 5×10^7 CFU of live GFP-expressing *S. aureus*. Mice were killed by CO_2 inhalation at 24 h after bacterial inoculation and the infiltrating inflammatory cells were isolated from the side of infection by extensively rinsing with warm DMEM medium. Inflammatory cells were incubated for 30 min with 20 μg/ml of lysostaphin to eliminate extracellular bacteria, incubated for 5 min at 4°C with anti-CD16/CD32 antibodies to block the FcR, followed by PE-conjugated anti-CD117 antibodies. After incubation for 30 min at 4°C, cells were washed and flow cytometry analysis was performed with an FACSCalibur™ (Becton Dickinson). Mast cells were gated according to their expression of CD117 antigen (FL2). Mast cells containing green-labeled staphylococci were identified by the expression of green fluorescence (FL1). In some experiments, pure fractions of mast cells (CD117+) were obtained by positive selection using magnetic beads (Miltenyi Biotec Inc., Bergisch-Gladbach, Germany) and MACS+ selection column (Miltenyi Biotec Inc.). Mast cells were then treated with lysostaphin to kill extracellular microorganisms, centrifuged and intracellular viable bacteria enumerated after disrupting the mast cells with 0.1% Triton X-100 in PBS.

The animal experiments were approved by the appropriate national ethics board (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany).

Statistical Analysis

Data analysis was performed by using Excel 2007 (Microsoft) or GraphPad Prism 5.0 (GraphPad Software). Each experiment was performed at least 3 times at independent occasions, and within each experiment samples were processed in triplicates. Comparison between groups was performed by the use of the Student's t test. p values of 0.05 or less were considered to be statistically significant.

Results

Phagocytosis-Independent Antimicrobial Activity of BMMC against S. aureus

To investigate the fate of *S. aureus* after encountering mast cells, *S. aureus* was cultured in the presence of BMMC at an MOI of 1:1 or in medium alone and the total amount of bacteria was determined in the culture supernatant at increasing times of co-culture. We found that BMMC exerted a vigorous antimicrobial activity against *S. aureus* that resulted in almost the complete radication of extracellular microorganisms (fig. 1a). The antimicrobial mechanism employed by BMMC to kill *S. aureus* was phagocytosis independent since addition of the phagocytosis inhibitor cytochalasin D did not affect the ability of BMMC to kill *S. aureus* (fig. 1a).

Because we have previously reported [13] that BMMC can release extracellular traps composed of DNA and granule proteins that ensnare and killed *Streptococcus pyogenes*, we next investigated the involvement of extracellular traps in the antimicrobial activity of BMMC against *S. aureus*. We observed that approximately 10% of BMMC released extracellular traps after 4 h of exposure to *S. aureus* (fig. 1c). The human mast cells HCM-1 were

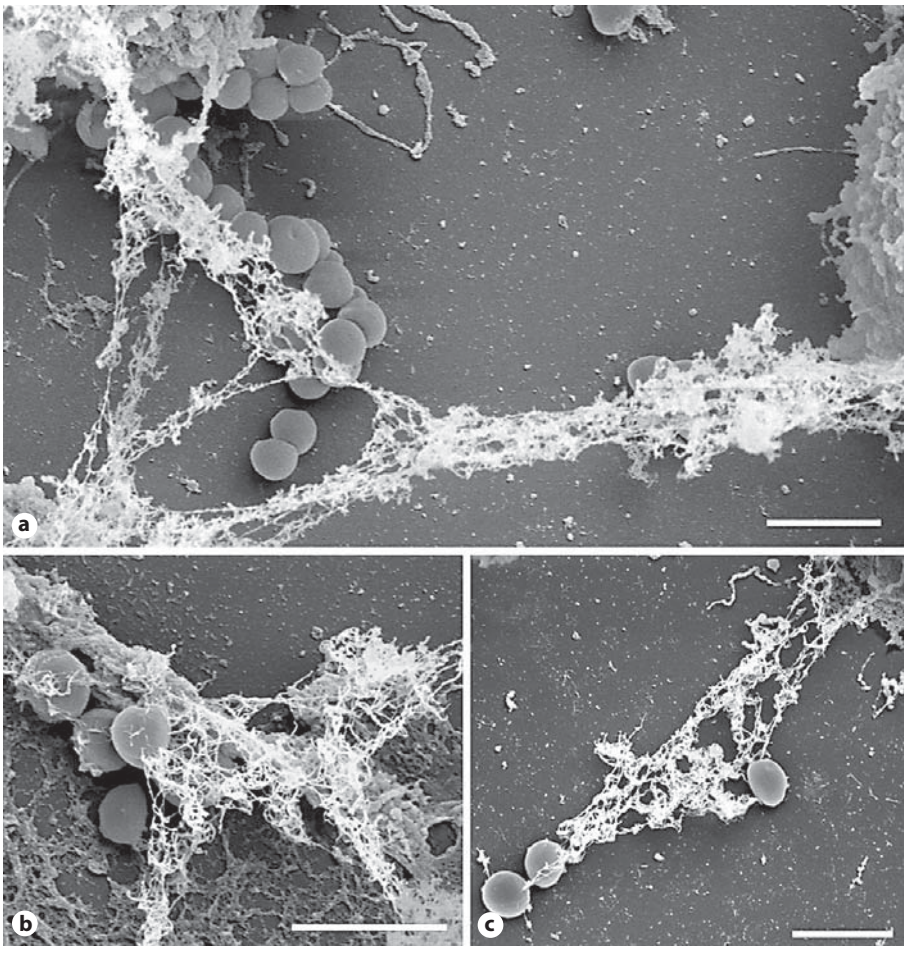

Fig. 2. Extracellular traps released by human HMC-1 following exposure to *S. aureus*. HMC-1 cells were seeded on poly-L-lysine coated glass slides, then infected with *S. aureus* for 4 h and fixed with 4% paraformaldehyde and examined by using field emission scanning electron microscopy. **a** Field emission scanning electron microscopy image of extracellular traps produced by HMC-1 during co-culture with *S. aureus*. **b**, **c** *S. aureus* microorganisms captured in the extracellular traps. Scale bars = 2 μm.

also capable of releasing extracellular traps in response to *S. aureus* infection (fig. 2). The extracellular traps released by the mast cells exerted an antimicrobial effect on *S. aureus* since staining with LIVE/DEAD bacterial viability dyes demonstrated that most entrapped microorganisms were dead (fig. 3a). In spite of the obvious involvement of these structures in the killing of *S. aureus*, this was not the only antimicrobial mechanism of mast cells since we found that the majority of extracellular staphylococci not associated with the extracellular traps were also killed (fig. 3b). These observations indicate that activation of mast cells by *S. aureus* involved not only the release of antimicrobial extracellular traps but also the discharge of antimicrobial compounds stored in the mast cell granules. Indeed, we demonstrated that mast cells underwent degranulation after encountering *S. aureus* as shown by the increasing concentrations of β-hexosaminidase (fig. 3c), tryptase (fig. 3d) and TNF-α (fig. 3e) in the supernatant of BMMC at progressive times of co-culture with *S. aureus*. In contrast to the rapid secretory events typical of anaphylactic degranulation evoked by the ligation of the FcεRI-receptor, mast cell degranulation following exposure to *S. aureus* was a gradual process and required longer time to reach completion (fig. 3c–e).

It has been reported that activated murine mast cells also synthesize and secrete peptides with potent antimicrobial activity such as the cathelicidin-related antimicrobial peptide (the murine homolog of human LL-37) [12]. In this regard, we found that filtered culture supernatant obtained from BMMC after 4 h of exposure to *S. aureus* exhibited a potent antimicrobial activity against this pathogen. In contrast, the supernatant from uninfected BMMC did not affect the bacterial viability or growth (fig. 4a). Furthermore, treatment with Cromolyn sodium, a stabilizing agent that inhibits mast cell degranulation or mediator release [19], significantly abolished the antimicrobial activity of BMMC against *S. aureus* (fig. 4b).

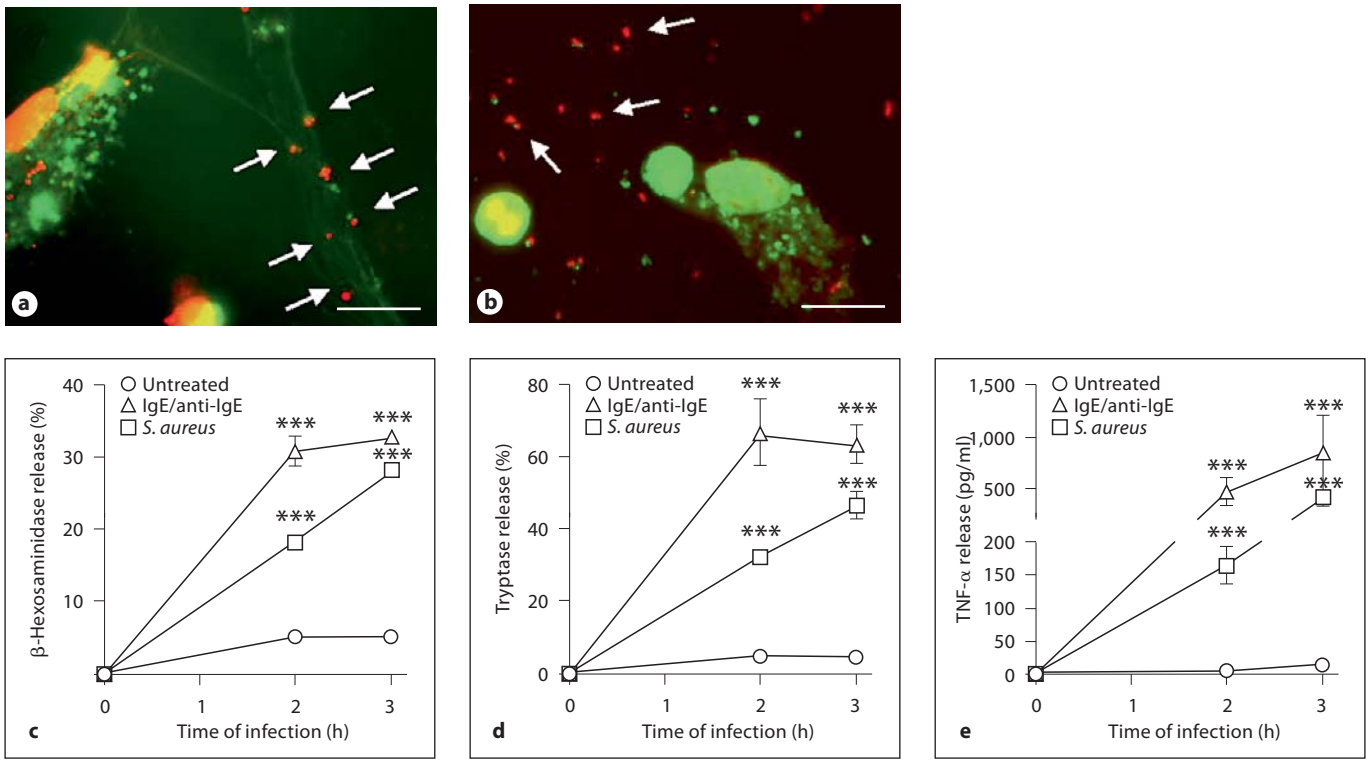

Fig. 3. Degranulation of BMMC in response to *S. aureus* infection. **a** Immunofluorescence photograph showing viable (green) versus dead (red) *S. aureus* ensnared in the extracellular traps (white arrows) released by the BMMC. Bacterial viability was determined using the LIVE/DEAD BacLight Bacterial Viability kit. **b** Immunofluorescence photograph showing viable (green) versus dead (red) *S. aureus* free in the extracellular milieu at 4 h of co-culture with BMMC. Bacteria are indicated by white arrows. Bacterial viability was determined using the LIVE/DEAD BacLight Bacterial Viability kit. ×220. Scale bars = 10 μm. Kinetic of β-hexosaminidase (**c**), tryptase (**d**) and TNF-α (**e**) release by BMMCs after aggregation of the high-affinity IgE receptor or during co-culture with *S. aureus*. Data represent means ± SD of 3 independent experiments performed in triplicate. *** $p < 0.001$ for treated versus untreated BMMC.

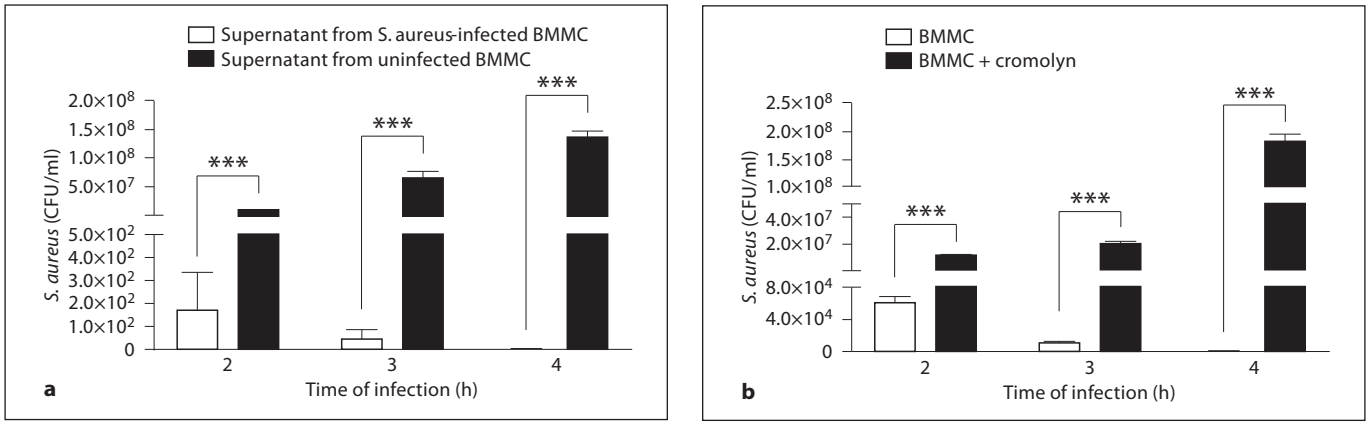

Fig. 4. The culture supernatant of *S. aureus*-activated BMMC exhibits potent antimicrobial activity. **a** Growth of *S. aureus* in culture supernatant obtained from untreated (black bars) or *S. aureus*-infected (white bars) BMMC. The supernatants of untreated *S. aureus*-infected (4 h) BMMC were filtered (0.2 μm) and used for *S. aureus* growth experiments. Data are expressed as CFU of *S. aureus* per milliliter of culture supernatant and each point represents the mean ± SD of 3 independent experiments. *** $p < 0.001$. **b** Growth of *S. aureus* in co-culture with either untreated or cromolyn-treated BMMC. Data are expressed as CFU of *S. aureus* per milliliter of culture supernatant. Each point represents the mean ± SD of triplicates from 3 independent experiments. *** $p < 0.001$.

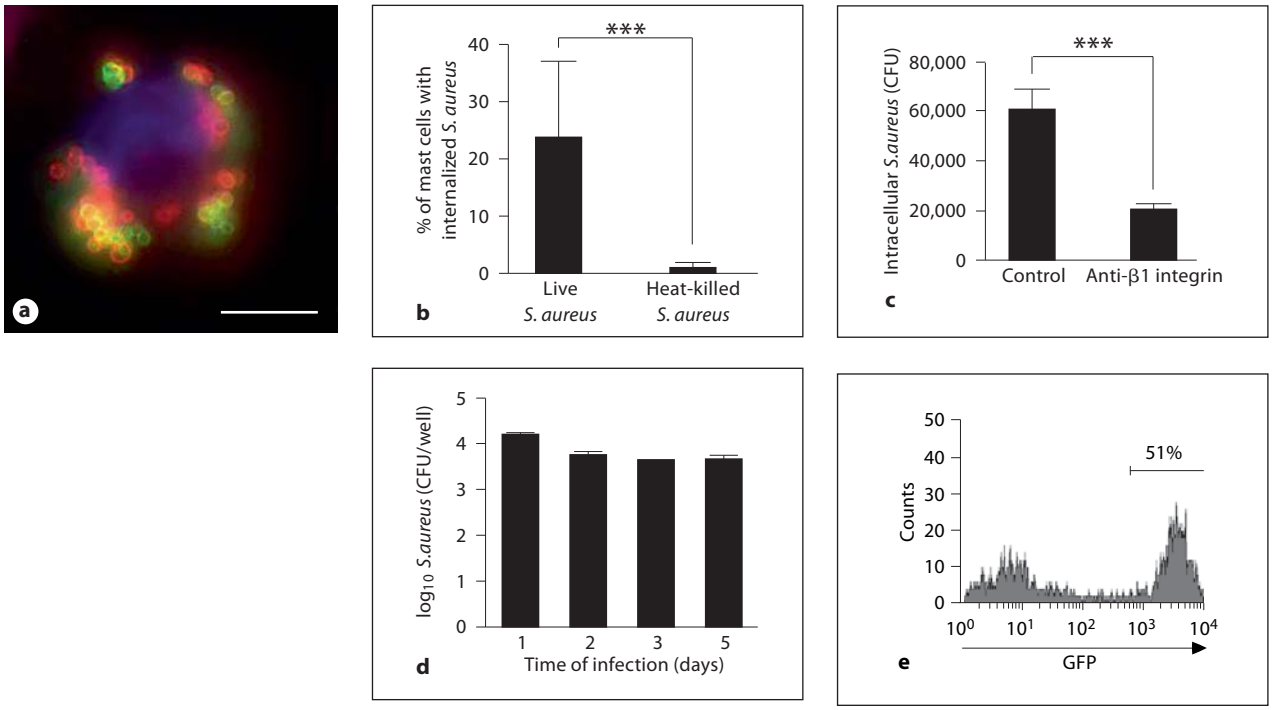

Fig. 5. *S. aureus* internalizes and persists within BMMC. **a** Double immunofluorescence staining of *S. aureus*-infected BMMC showing the intracellular/extracellular location of *S. aureus*. Extracellular bacteria are yellow-green and intracellular bacteria are red, DNA in the nucleus is stained in blue. Scale bar = 5 μm. **b** Quantification of live and heat-killed *S. aureus* internalized within BMMC. BMMC were exposed to viable or heat-killed *S. aureus* for 2 h and the amount of internalized bacteria was determined by immunofluorescence labeling. Data are presented as means ± SD of the percentage of BMMC with internalized bacteria determined in 10 different view fields and are representative of 3 independent experiments. *** $p < 0.001$. **c** Quantification of *S. aureus* internalized within untreated or of anti-β1 integrin antibodies-treated BMMC. BMMC were treated with anti-β1 integrin antibodies or left untreated, exposed to *S. aureus* for 2 h and extracellular bacteria eliminated by treatment with lysostaphin (20 μg/ml). The amount of viable intracellular *S. aureus* determined after serial plating. Each bar represents the mean ± SD of 3 independent experiments. *** $p < 0.001$. **d** Quantification of intracellular *S. aureus* in long-term cultured BMMC. BMMC were infected with *S. aureus* (MOI 20:1) for 2 h and extracellular bacteria eliminated by treatment with lysostaphin (20 μg/ml). After extensive washing, BMMC were resuspended in medium containing gentamicin (100 μg/ml), harvested at progressive times of infection, disrupted and the amount of viable intracellular *S. aureus* determined after serial plating. Each bar represents the mean value ± SD of 3 independent experiments. *** $p < 0.001$. **e** Histogram analysis showing in vivo internalization of *S. aureus* within skin mast cells. Mice were subcutaneously inoculated with either 5×10^7 live GFP-expressing *S. aureus*, infiltrating cells were isolated from the side of bacterial inoculation at 24 h of infection and the amount of mast cells (CD117+) containing *S. aureus* was determined by flow cytometry. The percentage of mast cells containing *S. aureus* was determined by the increase in green-fluorescence (FL1) within the gated CD117+ cell population. One representative experiment out of 3 is shown.

Taken together, these observations indicate that the mast cell response to *S. aureus* challenge is multifactorial and involves both the release of extracellular traps as well as the secretion of compounds with potent antimicrobial activity.

S. aureus *Internalizes within BMMC to Subvert Their Extracellular Antimicrobial Activity*

It has been reported that certain pathogens have evolved mechanisms to subvert the antimicrobial mechanisms of mast cells. These include the intracellular survival after bacterial phagocytosis and the production of toxins that inhibit the release of mediators by the mast cell [9]. As it has been previously reported that *S. aureus* is capable to internalize within human umbilical mast cells [20], we investigated the capacity of *S. aureus* to internalize within BMMC as potential mechanism to escape the extracellular antimicrobial activity of these cells. *S. aureus* was capable to internalize within BMMC (fig. 5a) and approximately 20% of BMMC in culture con-

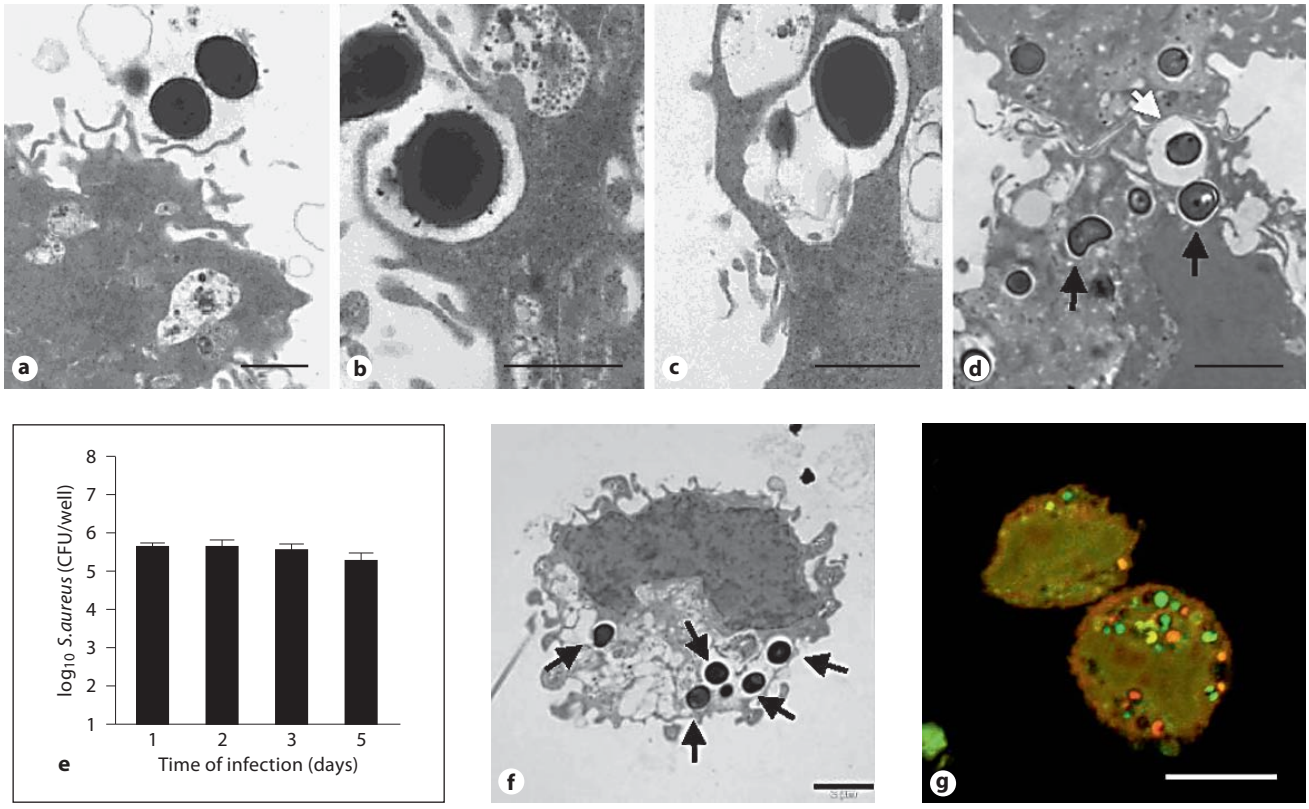

Fig. 6. *S. aureus* internalizes and persists within HMC-1. **a–d** Transmission electron photographs showing adhesion (**a**), engulfment (**b**) and internalization (**c**) of *S. aureus* within HMC-1 cells. Intracellular *S. aureus* can be found residing in a large membrane bound compartment at the cell periphery (**d**, white arrow) or in smaller/tighter membrane bound vacuole near the nucleus (**d**, black arrows). Scale bars = 1 μm (**a–c**). Scale bars = 2 μm (**d**). **e** Quantification of intracellular *S. aureus* in long-term cultured HMC-1. HMC-1 cells were infected with *S. aureus* (MOI 20:1) for 2 h and extracellular bacteria eliminated by treatment with lysostaphin (20 μg/ml). After extensive washing, HMC-1 cells were resuspended in medium containing gentamicin (100 μg/ml) and further incubated at 37°C. HMC-1 cells were then harvested after different periods of time, disrupted and the amount of viable intracellular *S. aureus* determined after serial plating. Each bar represents the mean value ± SD of 3 independent experiments. **f** Transmission electron photograph showing *S. aureus* (black arrows) internalized within HMC-1 cells at day 5 of infection. Scale bar = 2 μm. **g** Immunofluorescence photograph showing viable (green) versus dead (red) staphylococci within HMC-1 cells at day 5 of infection. Bacterial viability was determined using the LIVE/DEAD BacLight Bacterial Viability kit. ×220. Scale bar = 5 μm.

tained intracellular *S. aureus* after 2 h of infection (fig. 5b). Bacterial internalization seems to be an active process promoted by the staphylococci since it requires bacterial viability (fig. 5b). Since the most important molecular mechanism used by *S. aureus* to invade eukaryotic cells has been reported to be mediated via fibronectin bridging between α5β1 integrin in the host cells and fibronectin-binding proteins on the bacterial surface [21], we evaluated the effect of disrupting the fibronectin-α5β1 integrin association using anti-β1 integrin antibodies in the internalization of *S. aureus* within BMMC. Staphylococci internalization was significantly reduced but not completely abolished after treatment with anti-β1 integrin antibodies (fig. 5c). These results indicate that the fibronectin-binding protein-fibronectin-α5β1 integrin signaling pathway is indeed important for staphylococci internalization, but they also suggest the presence of additional pathways used by *S. aureus* for internalizing within the BMMC. After internalization, a significant fraction of *S. aureus* microorganisms survived and persisted for long periods within the BMMC (fig. 5d).

We also demonstrated the ability of *S. aureus* to internalize within murine mast cells during in vivo infection. For this purpose we used a murine model of staphylococcal skin infection. In this model, mice were injected subcutaneously with viable GFP-expressing *S. aureus* and the inflammatory cells recruited to the site of infection were collected at 24 h after bacterial inoculation. Infil-

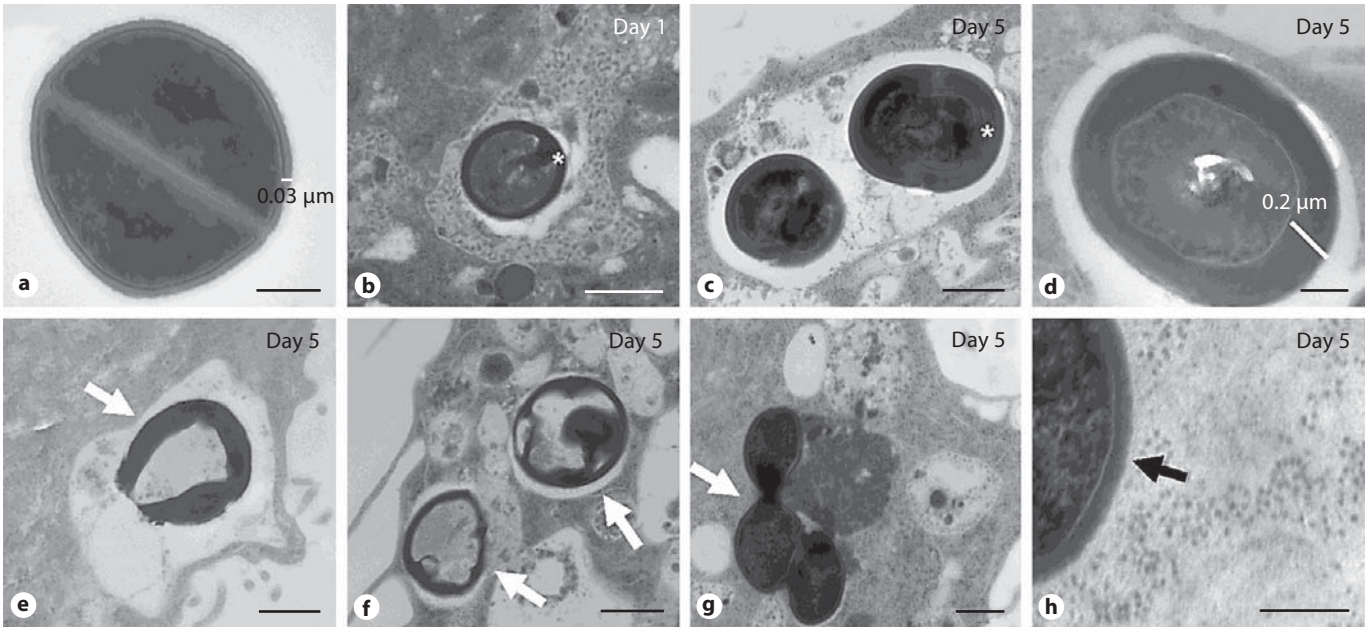

Fig. 7. Intracellular *S. aureus* undergoes morphological changes and resides within the mast cell cytosol. **a** Transmission electron photograph of *S. aureus* grown in medium. The size of the cell wall was determined by morphometric analysis (0.03 μm). **b–d** Transmission electron photographs showing intracellular *S. aureus* at day 1 (**b**) and day 5 (**c**) of infection. The bacterial cell wall is indicated by an asterisk. (**c**) Morphometric analysis of the cell wall of intracellular *S. aureus* at day 5 of infection (0.2 μm). **e**, **f** Transmission electron photographs showing partially degraded intracellular staphylococci (white arrows). **g** *S. aureus* undergoing cell division within the mast cell cytosol at day 5 after bacterial internalization (white arrow). **h** *S. aureus* residing in the mast cell cytosol (black arrow). Scale bars = 0.2 μm (**a**, **d**, **h**). Scale bars = 0.5 μm (**b**, **c**, **e–g**).

trating cells were treated with lysostaphin for 30 min to lyse extracellular staphylococci and stained with PE-conjugated anti-CD117 antibodies to identify the mast cell population by flow cytometry. The results showed that approximately 50% of the mast cells isolated from the skin of infected mice contained GFP staphylococci (fig. 5e). Furthermore, quantification of the intracellular bacteria after disrupting the mast cells evidenced that a proportion of the intracellular staphylococci (approx. 2×10^3 *S. aureus* per 10^5 mast cells) were viable.

Intracellular S. aureus *Survives for Long Periods within Human Mast Cells*

Similarly to BMMC, *S. aureus* was also capable not only to internalize but also to survive for prolonged periods of time within human HMC-1. Electron microscopy photographs depicted in fig. 6a show the ability of *S. aureus* to adhere to the surface of HMC-1 cells (fig. 6a). Surface-attached staphylococci were then engulfed (fig. 6b) and internalized (fig. 6c) within the HMC-1 cells. Intracellular bacteria reside in either large membrane-bound compartments (fig. 6d, white arrow) or in a smaller/tighter membrane-bound vacuole (fig. 6d, black arrows). Quantification of viable intracellular staphylococci at days 1, 2, 3 and 5 of infection demonstrated that the amount of intracellular organisms remained relatively stable during this 5-day period (fig. 6e). The electron microscopy photograph depicted in fig. 6f illustrates the intracellular staphylococci at day 5 of infection. The viability of a proportion of internalized *S. aureus* was further demonstrated by LIVE/DEAD bacterial viability dyes (live bacteria with intact membranes fluoresced green, while dead bacteria with damaged membranes fluoresced red) (fig. 6g).

Survival Strategies Used by Staphylococci to Persist for Long Periods within Mast Cells

An interesting observation arising from the electron microscopy examination of intracellular staphylococci was the time-dependent changes in the bacteria morphology. At 24 h of infection, intracellular microorganisms (fig. 7b) were very similar in appearance to those initially used for infecting the mast cells (fig. 7a). At day 5 of infection, intracellular *S. aureus* exhibited signifi-

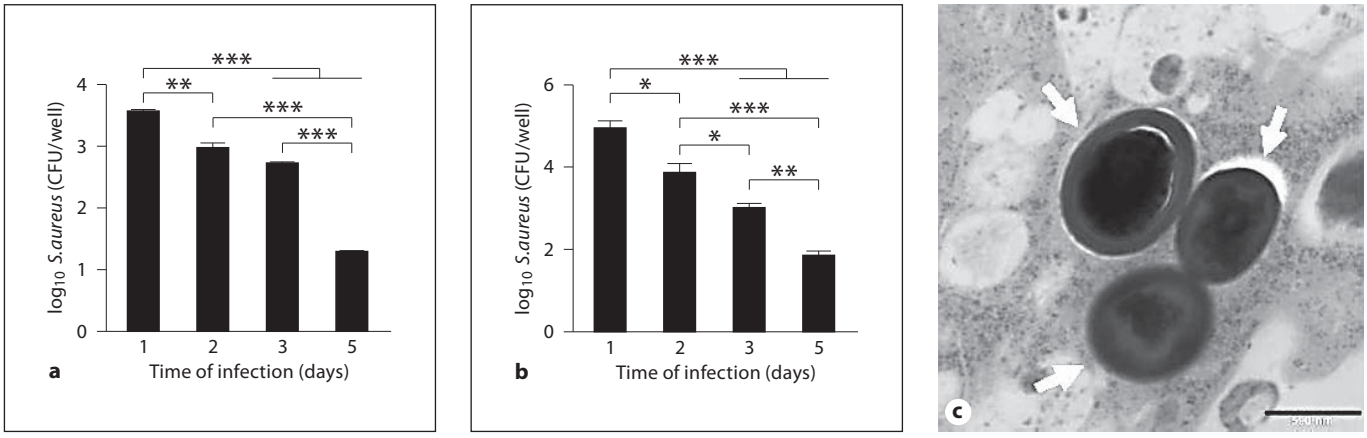

Fig. 8. The expression of α-hemolysin is important for *S. aureus* intracellular survival. **a**, **b** Quantification of intracellular *S. aureus* hla$^{-/-}$ in long-term cultured BMMC (**a**) or HMC-1 cells (**b**). BMMC or HMC-1 cells were infected with this strain of *S. aureus* (MOI 20:1) for 2 h and extracellular bacteria eliminated by treatment with lysostaphin (20 μg/ml). After extensive washing, cells were resuspended in medium containing gentamicin (100 μg/ml) and further incubated at 37°C. The cells were then harvested after different periods of time, disrupted and the amount of viable intracellular *S. aureus* determined after serial plating. Each bar represents the mean value ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. **c** Transmission electron photograph showing *S. aureus* hla$^{-/-}$ (white arrows) residing in the mast cell cytosol at day 5 of infection. Scale bar = 0.5 μm.

cantly increased cell wall thickness (fig. 7c, asterisk) and contraction of the bacterial cytoplasm (fig. 7c). The size of the cell wall was determined by morphometric analysis and estimated to be 0.03 μm for *S. aureus* microorganisms grown in broth (fig. 7a) and between 0.14 and 0.23 μm for intracellular *S. aureus* at day 5 of infection (fig. 7d). Some of the internalized organisms underwent cell death (fig. 7e, f, white arrows) while others underwent cell division (fig. 7g, white arrow). The ultrastructural image depicted in fig. 7h demonstrated that the actively dividing staphylococci were free in the cytoplasm of the mast cell (fig. 7h, black arrow). These observations indicate that the survival of *S. aureus* within mast cells could be facilitated by its ability to breach the vacuolar compartment gaining access into the nutrient-rich mast cell cytosol.

Expression of α-Hemolysin Is Critical for S. aureus *Intracellular Persistence within Mast Cells*

Some reports have suggested the involvement of staphylococcal α-hemolysin in phagolysosomal escape in cystic fibrosis tracheal epithelial cell line CFT-1 [22]. To determine the relevance of α-hemolysin expression for the survival of *S. aureus* within BMMC (fig. 8a) or HMC-1 (fig. 8b), we determined the time-dependent intracellular survival of an isogenic *S. aureus* mutant strain deficient in the production of α-hemolysin (hla$^{-/-}$). *S. aureus* hla$^{-/-}$ exhibited an impaired capacity to survive within BMMC (fig. 8a) or HMC-1 (fig. 8b) as shown by the progressive decrease in the number of viable bacteria recovered at increasing incubation times. Electron microscopy examination of the few microorganisms remaining within the mast cell after 5 days of infection evidenced that, like the wild-type strain, hla$^{-/-}$ staphylococci were free in the cell cytosol and capable to increase the size of the cell wall (fig. 8c, white arrows). These results indicate that α-hemolysin is important for *S. aureus* intracellular survival but they also suggest that α-hemolysin is not required for *S. aureus* to gain access into the mast cell cytosol.

Discussion

Mast cells are key elements of the immune system ideally positioned throughout the body gateways, such as the skin and mucosal surfaces, so that they can encounter invading pathogens early in the course of an infection. Several reports have supported an important function of mast cells promoting innate immunity against pathogens [7–9]. In this study, we have performed an in-depth characterization of the interactions of *S. aureus,* one of the most important human pathogens, with primary BMMC as well as the human mast cell line HMC-1. Our results demonstrated that BMMC became activated after encountering *S. aureus* and exerted a direct antimicrobial activity against this pathogen that was mediated by both

the release of extracellular traps as well as the discharge of antimicrobial products that are toxic for *S. aureus* (such as the cathelicidin-related antimicrobial peptide) [12]. Another important effector function of mast cells in the context of *S. aureus* infection was the release of mediators such as TNF-α, which serve to attract phagocytic cells involved in the elimination of this invading pathogen to the site of infection. The additive effect of these two mechanisms (direct antimicrobial activity and release of inflammatory mediators) may contribute, at least to some extent, to the early control of *S. aureus* infection.

There is a growing realization that a variety of immune cells are infected by the very pathogens that they are supposed to combat. This may constitute a pathogenic strategy to escape the extracellular antimicrobial actions since, in the privileged intracellular location, the pathogens are protected from the bactericidal compounds released by the immune cells. In this regard, there is ample evidence that *S. aureus*, despite being considered an extracellular pathogen, can also invade several cell types in culture including endothelial cells, epithelial cells, fibroblasts, osteoblasts, keratinocytes, professional phagocytes and human umbilical mast cells [20, 23–25]. Here, we demonstrated that *S. aureus* was also capable to internalize within BMMC and HMC-1 cells in culture and within skin mast cells during in vivo infection. We noted that viable *S. aureus* was much more efficiently internalizing within mast cells than heat-killed bacteria, suggesting that *S. aureus* directs its own uptake. Thus, gaining access to an intracellular compartment may constitute a strategy of *S. aureus* to circumvent the extracellular killing activity of mast cells. The most prominent molecular mechanism used by *S. aureus* to invade eukaryotic cells has been reported to be via fibronectin bridging between host α5β1 integrins and staphylococcal surface fibronectin-binding proteins FnBPA and FnBPB [21]. This seems to be an important mechanism for the invasion of mast cells by *S. aureus* since we observed that disruption of the fibronectin-α5β1 integrins association using anti-β1 integrin antibodies resulted in significant reduction in the amount of internalized bacteria. Nevertheless, staphylococcal internalization was not completely inhibited by the anti-β1 integrin treatment implying that the mechanism used by staphylococci to gain access into mast cells is multifactorial and other bacterial invasines may also be involved. Support for a multifactorial mechanism is provided by a previous study reporting the involvement of CD48 and TLR2 signaling in *S. aureus* internalization within human cord blood-derived mast cells [20].

Once inside the mast cell, *S. aureus* not only survived but also persisted for prolonged periods of time. Pathogens that survive and replicate within host cells possess virulence mechanisms that enhance their survival. *Salmonella* and *Legionella* use mechanisms that prevent their targeting to the phagosomelysosome fusion pathway [26]. *Shigella flexneri* and *Listeria monocytogenes*, in contrast, have the ability to break out of the phagocytic vesicle to reside and replicate in the nutrient-rich host cell cytosol [26]. In serum-deficient conditions, the type-1 fimbriated strains of *Escherichia coli* gain access into mast cells without the loss of bacterial viability by a route distinct from the classical endosome-lysosome pathway [27]. In the particular case of *S. aureus*, phagolysosomal escape has been observed by several groups in different cell types [22, 28]. In line with these reports, we found that *S. aureus* was capable to breach the vacuolar compartment within the mast cell gaining access into the cell cytosol.

The molecular mechanism used by *S. aureus* to breach the phagocytic vacuole and gain access into the cytoplasm of the eukaryotic cell remains rather controversial. Some reports have suggested the involvement of staphylococcal α-hemolysin, a pore-forming toxin that is secreted as a soluble monomer and forms heptameric transmembrane pores in target cell membranes [29], in phagolysosomal escape [22]. Other studies, however, argued against a primary role of α-hemolysin in this process [30]. Furthermore, a recent study has shown the synergistic activity of the cytolytic peptide, staphylococcal δ-toxin and the sphingomyelinase β-toxin enabling the phagosomal escape of *S. aureus* in human epithelial and in endothelial cells [31]. In our experimental settings, we found that the expression of α-hemolysin was critical for *S. aureus* persistence within the mast cells but we also found that *S. aureus* deficient in the production of α-hemolysin retained the ability to escape the phagocytic vacuole and to gain access into the mast cell cytosol. These observations indicate that the functional activity of α-hemolysin in promoting staphylococci intracellular survival is other than facilitating the bacterial escape from the vacuolar compartment. Interestingly, a recent study has identified disintegrin and metalloprotease 10 (ADAM10) as the probable high-affinity cellular receptor for α-hemolysin in eukaryotic cells [32]. Upon binding, the complex α-hemolysin/ADAM10 relocalizes to lipid rafts that serve as a platform for the clustering of signaling molecules [32]. Therefore, the possibility that *S. aureus* utilizes α-hemolysin to gain access into mast cells by a pathway that favors bacterial survival rather than degradation warrants further studies.

Another important finding of this study is the notable increase in cell wall thickness of *S. aureus* during intracellular persistence. Exposure of *S. aureus* to a broad range of cell wall-damaging agents has been shown to trigger the induction of a cell wall stress stimulon (CWSS), controlled by the vancomycin resistance-associated sensor/regulator (vraSR) two-component system [33]. Activation of the CWSS is predicted to enhance cell wall synthesis since clinical isolates with point mutations in the *vraSR* operon exhibit increased basal expression of the CWSS in the absence of inducing agents and thickened cell walls [34]. Therefore, it can be speculated that the increase in the cell wall thickness observed in staphylococci during intracellular persistence in mast cells may be the result of the bacterial transcriptional adaption to the intracellular milieu in response to potentially cell wall harmful cellular activities (such as oxidative stress). In this regard, upregulation of cell wall and techoic acid synthesis genes has been observed in *S. aureus* following internalization within human lung epithelial cells [35]. Furthermore, in a recent publication reporting the in vivo proteome profile of *S. aureus* internalized within human bronchial epithelial cells, Schmidt and co-workers [36] showed that, of the 17 potential two-component systems identified in *S. aureus*, only the response regulator VraR increased in level over time after internalization into host cells. The transcriptional response of *S. aureus* during intracellular persistence within the mast cell is currently under investigation.

Interestingly, the clinical isolates with point mutations in the *vraSR* operon exhibiting thickened cell walls also displayed increased levels of resistance to glycopeptide and β-lactam [34, 37]. This observation suggests that a thickened cell wall may serve as a physical barrier inhibiting the penetration of antibiotics to its active site within the bacterial cell. Therefore, thickening of the cell wall may not only be used by *S. aureus* to resist elimination by the intracellular antimicrobial mechanisms but it may also confer protection against antibiotic eradication.

By hiding within the long-life mast cells, *S. aureus* not only avoids clearance but also establishes an infection reservoir that could contribute to chronic carriage. This can be of particular relevance for patients affected by atopic dermatitis (AD), a chronic inflammatory skin disease that affects a large percentage of the world's population. AD patients exhibit significantly higher rates of *S. aureus* carriage in the skin (77–96%) compared with only 5–10% of healthy individuals [38]. As the number of mast cells in chronic AD lesions is significantly increased, especially in areas of lymphocytic infiltration in the papillary dermis [39], it is very likely that mast cells may serve as reservoir of viable staphylococci in AD patients and can explain their high *S. aureus* carriage rate. Persistent antibiotic treatment in an attempt to eradicate *S. aureus* colonization in AD patients may eventually lead to failure of these antibiotics in the setting of invasive infections, which are not uncommon in AD patients [40]. Thus, it is crucial to obtain additional information on the function and importance of molecular pathways that play a critical role on *S. aureus* survival within the mast cell. This will enable the development of novel therapeutic strategies to optimize *S. aureus* eradication while reducing the potential for antimicrobial drug resistance.

Acknowledgments

The authors wish to thank Joseph H. Butterfield (Mayo Foundation for Medical Education and Research, Rochester, Minn., USA) for providing HMC-1 cells and Ina Schleicher for excellent technical assistance. Financial support for this study was provided in part by the DFG-Deutsche Forschungsgemeinschaft, SPP 1394: 'Mast cells – promoters of health and modulators of disease', project ME 1875/2-1 and in part by the BMBF – Suszeptibilität bei Infektionen: SkinStaph (FKZ 01KI1009B).

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