The Janus Face of α -Toxin: A Potent Mediator of Cytoprotection in Staphylococci-Infected Macrophages

Joanna Koziel^{a, b} Daniela Chmiest^{a, b} Danuta Bryzek^{a, b} Katarzyna Kmiecik^{a, b}
Danuta Mizgalska^{a, b} Agnieszka Maciag-Gudowska^{a, b} Lindsey N. Shaw^c
Jan Potempa^{a, b, d}

^aDepartment of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology and ^bMalopolska Center of Biotechnology, Jagiellonian University, Krakow, Poland; ^cDepartment of Cell Biology, Microbiology and Molecular Biology, University of South Florida, Tampa, Fla., and ^dDepartment of Oral Immunology and Infectious Diseases, University of Louisville School of Dentistry, Louisville, Ky., USA

Key Words

α -Toxin · Macrophages · *Staphylococcus aureus* infection · Cytoprotection · MCL-1 · Gene regulation

Abstract

After phagocytosis by macrophages, *Staphylococcus aureus* evades killing in an α -toxin-dependent manner, and then prevents apoptosis of infected cells by upregulating expression of antiapoptotic genes like *MCL-1* (*myeloid cell leukemia-1*). Here, using purified α -toxin and a set of *hla*-deficient strains, we show that α -toxin is critical for the induction of *MCL-1* expression and the cytoprotection of infected macrophages. Extracellular or intracellular treatment of macrophages with α -toxin alone did not induce cytoprotection conferred by increased Mcl-1, suggesting that the process is dependent on the production of α -toxin by intracellular bacteria. The increased expression of *MCL-1* in infected cells was associated with enhanced NF κ B activation, and subsequent IL-6 secretion. This effect was only partially inhibited by blocking TLR2, which suggests the participation of intracellular receptors in the specific recognition of *S. aureus* strains secreting α -toxin. Thus, *S. aureus* recognition by intracellular receptors and/or activation of downstream pathways lead-

ing to Mcl-1 expression is facilitated by α -toxin released by intracellular bacteria which permeabilize phagosomes, ensuring pathogen access to the cytoplasmatic compartment. Given that the intracellular survival of *S. aureus* depends on α -toxin, we propose a novel role for this agent in the protection of the intracellular niche, and further dissemination of staphylococci by infected macrophages.

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Introduction

Staphylococcus aureus is famous for its ability to cause frequent and severe nosocomial and community-acquired infections in humans, which are largely attributable to the plethora of virulence factors it produces. Perhaps the most important amongst these is α -toxin (Hla), which is a crucial virulence factor that is produced by the majority of *S. aureus* strains [1]. α -Toxin is a 33-kDa polypeptide that functions as a cell membrane pore-forming toxin [2], exerting cytolytic, dermonecrotic and lethal activities [3, 4]. Isogenic *hla*-deficient mutants are significantly less virulent than parental strains [5]. Furthermore, in epithelial cells, α -toxin is required for *S. au*-

reus escape from endocytic vesicles into the cytosol [6]. In macrophages, α -toxin-dependent evasion of phagosomes promotes resistance to killing by the phagocyte, as determined by TEM imaging of phagosome membrane damage [7]. At the same time, an increasing body of evidence indicates that α -toxin sensing by the host is important for triggering an immune response [8], and contributes to host defense against systemic infection by staphylococci [9, 10]. Indeed, *S. aureus* sensing by innate immunity is mediated mainly via Toll-like receptors (TLRs), and the group of nucleotide-binding oligomerization domain (NOD)-like receptors [11] that detect bacterial antigens in the cytoplasm. Recently, we showed that live intracellular staphylococci induce cytoprotection in human macrophages to promote survival of infected cells without bacterial eradication, allowing the pathogen to silently persist and remain invisible to the immune system [7, 12]. Moreover, we proposed that the pathogen induces a prosurvival signaling pathway through the induction of expression of antiapoptotic factors, including myeloid cell leukemia-1 (Mcl-1), an antiapoptotic protein of the Bcl-2 family [13]. In this context, the physiological significance of α -toxin interaction with detection systems associated with innate immunity for the survival of *S. aureus* in infected macrophages has not yet been elucidated.

The results of this study demonstrate a novel mechanism of dependence for Mcl-1 expression on α -toxin production by bacteria, since Hla producers, in comparison to strains deficient in this factor, induced the highest levels of Mcl-1 and cytoprotection. Furthermore, we established that α -toxin-mediated induction of Mcl-1 expression required the IL-6 and NF κ B transcription pathway. Mcl-1 expression in infected macrophages was largely independent of extracellular TLR2 signaling, so we propose that the observed induction of expression is the result of intracellular receptor(s) activation.

Materials and Methods

Reagents

Gentamycin, L-glutamine, DEVD-AFC peptide substrate, SYBR Green JumpStart Taq Ready Mix, erythromycin, tetracycline, RNase A, proteinase K, bovine serum albumin, tryptic soy agar, tryptic soy broth, staurosporine, staphylococcal α -toxin protein and FITC were from Sigma (Saint Louis, Mo., USA). Fetal calf serum, RPMI 1640, DMEM, phosphate-buffered saline (PBS; without Ca²⁺ and Mg²⁺) and lymphocyte separation medium were obtained from PAA (Pasching, Austria). The Pro-Ject protein transfection kit was from Pierce (Rockford, Ill., USA). Rabbit polyclonal anti-human Mcl-1 antibodies were purchased from Santa

Table 1. *S. aureus* strains

<i>S. aureus</i> strains	Relevant genotype/markers	Relevant properties	Source
Newman		WT strain	T.J. Foster
Newman (<i>hla</i> ⁻)	<i>hla</i> ::Erm ^R	<i>hla</i> ⁻ ; mutant of Newman	T.J. Foster
USA300		WT strain	L. Shaw
USA300 (<i>hla</i> ⁻)	<i>hla</i> ::Erm ^R	<i>hla</i> ⁻ ; mutant of USA300	L. Shaw
SH1000		WT strain	L. Shaw
SH1000 (<i>hla</i> ⁻)	<i>hla</i> ::Erm ^R	<i>hla</i> ⁻ ; mutant of SH1000	L. Shaw

Cruz Biotechnology (Dallas, Tex., USA) and mouse monoclonal anti- β -actin from Sigma. *S. aureus*-derived peptidoglycan (PGN) was from Invitrogen (Carlsbad, Calif., USA). Secondary horseradish peroxidase (HRP)-conjugated antibodies, donkey anti-rabbit IgG and sheep anti-mouse IgG were obtained from Amersham Biosciences (Freiburg, Germany) and Sigma, respectively. The neutralizing antibodies against human IL-6 receptor (IL-6R) were from R&D Systems. TLR2 [TL2.1] and TLR4 [HTA125] were from Abcam (Cambridge, Mass., USA).

Cell Culture

Human monocyte-derived macrophages (hMDMs) were differentiated from peripheral blood mononuclear cells as described previously [12]. Blood was obtained from the Red Cross, Krakow, Poland. The Red Cross deidentifies blood materials, as is appropriate for human subject confidentiality assurances. This study therefore did not require consent from any patients or approval by any IRB. Briefly, PBMCs were isolated from human blood using a lymphocyte separation medium density gradient, yielding a fraction highly enriched in monocytes (90% CD14-positive), as described previously. Cells were plated at 3×10^6 /well in 24-well plates (Sarstedt, Numbrecht, Germany) in RPMI 1640 supplemented with 2 mM L-glutamine, 50 μ g/ml gentamycin, and 10% autologous human serum. After 24 h, nonadherent PBMCs were removed by washing with complete medium, and adherent cells were cultured in this medium for 7 days, with media changed every 2 days. The murine macrophage cell line RAW 264.7 obtained from the American Type Culture Collection was maintained in DMEM supplemented with 5% fetal bovine serum.

Bacterial Strains, Storage and Growth Conditions

Laboratory *S. aureus* strains (from lab stocks, as well as kindly provided by Dr. T.J. Foster, Trinity College, Dublin, Ireland) used in this study are listed in table 1. The strains were stored in tryptic soy broth medium containing glycerol (50% v/v) at -80°C . Cultures were inoculated from stocks into 10 ml of media. *S. aureus* strains were grown to stationary phase at 37°C overnight, with shaking (180 rpm). If required, media were supplemented with antibiotics at the following concentration: erythromycin at 5 μ g/ml or tetracycline at 2 μ g/ml. Bacterial cells were collected by cen-

trifugation (5,000 g for 8 min), washed with PBS and resuspended in PBS to the desired optical density (OD)₆₀₀. The numbers of vital bacteria in samples used for phagocytosis assay were routinely verified by plating dilutions on agar plates and counting colonies to determine CFUs/ml. Heat treatment (80°C for 1 h) was used to kill bacteria.

Infection of Macrophages

To infect macrophages, phagocytosis with *S. aureus* was carried out for 2 h at 37°C at a multiplicity of infection (MOI) of 1:50 (hMDMs) or 1:5 (RAW 264.7). This resulted in >85% of macrophages engulfing at least one bacterium, as verified by plating cell lysates and counting colonies to determine CFUs. Infected cells were rinsed 4 times with ice-cold PBS, and any remaining non-phagocytosed bacteria were killed by culturing in medium containing gentamycin (50 µg/ml). The ability of intracellular *S. aureus* to form colonies was determined 2, 6, 24 and 48 h after the infection of macrophages by plating cell homogenates on agar and counting colonies (in CFUs). For toxin supplementation experiments, α -toxin or bacterial culture medium (0.3%) was added simultaneously with *S. aureus*. Blocking experiments for IL-6R, TLR2 and TLR4 were performed with anti-IL-6R, anti-TLR2 and anti-TLR4 monoclonal antibodies (mAbs). Briefly, noninfected cells were incubated with 1–5 µg/ml of each mAb for 30 min at 37°C, before the macrophages were infected with bacteria and the effects on cells were evaluated.

Analysis of Caspase-3 Activity

The activity of caspase-3, a main executioner caspase involved in the apoptotic process, was determined by release of AFC (7-amino-4-trifluoromethyl-coumarin) from a DEVD-AFC peptide substrate. Both control cells and those exposed to *S. aureus*, with or without apoptotic stimuli, were collected by centrifugation (200 g for 5 min at 4°C), washed with ice-cold PBS and resuspended in 100 µl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS). Samples were then incubated on ice for 20 min and subjected to centrifugation (16,000 g for 10 min). The protein content of supernatants was measured using the BCA method. Caspase activity was determined by transferring aliquots of supernatant into buffer (40 mM Pipes, 20% sucrose, 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA) containing DEVD-AFC and recording increased fluorescence (λ_{ex} = 350 nm, λ_{em} = 460 nm) for released AFC using a Spectra Max Gemini EM (Molecular Devices). Additionally, processing of 32 kDa procaspase-3 to its active form (20-kDa and 11 kDa chains) was determined in cells by Western blot.

Protein Isolation and Immunoblotting

Whole-cell extracts from control and *S. aureus*-exposed macrophages were prepared from cells detached with a rubber policeman, and harvested from culture medium into microfuge tubes. Cells were washed twice with PBS (250 g for 5 min), resuspended in 100 µl of RIPA-lysis buffer (0.25% Na-deoxycholate, 0.5% Nonidet P-40, 0.05% SDS, protease inhibitor cocktail, 2.5 mM EDTA in PBS) and stored at –20°C. Equal amounts of protein (40 µg/well) were separated by SDS-PAGE (8, 12 or 16% gels depending on the molecular mass of proteins of interest) and electrotransferred onto nitrocellulose membranes (BioRad) in buffer composed of 25 mM Tris, 0.2 M glycine, 20% methanol (30 V, overnight). Membranes were stained with Ponceau S to analyze the

Table 2. Oligonucleotide sequences used in qRT-PCR

Oligonucleotide	Annealing	Sequence
EF-2 F	62°C	5'-GACATCACCAAGGGTGTGCAG-3'
EF-2 R	62°C	5'-TCAGCACACTGGCATAGAGGC-3'
IL-6 F	54°C	5'-CATCTTTGGAAGGTTTCAGGTTTGT-3'
IL-6 R	54°C	5'-AGCCCTGAGAAAGGAGACATGTA-3'
MCL1 F	62°C	5'-TAAGGACAAAACGGGACTGG-3'
MCL1 R	62°C	5'-ACCAGCTCCTACTCCAGCAA-3'
NOD2 F	56°C	5'-CTGCAAGGCTCTGTATTTGC-3'
NOD2 R	56°C	5'-CTCGCAGTGAAGAGCACATT-3'
Hla F	55°C	5'-ATTTGCACCAATAAGGCCGC-3'
Hla R	55°C	5'-TGGTTTAGCCTGGCCTTCAG-3'
16S F	55°C	5'-GCTGCCCTTTGTATTGTC-3'
16S R	55°C	5'-AGATGTTGGGTTAAGTCCC-3'

efficiency of transfer and loading precision (same amount of protein loaded per well). Nonspecific binding sites were blocked with 3% bovine serum albumin in TTBS buffer (20 mM Tris, 0.5 M NaCl, pH 7.5 with 0.05% Tween 20) for 1 h, followed by incubation for 1–2 h with the relevant primary antibody: 100-fold diluted anti-Mcl-1 or 3,000-fold diluted anti- β -actin. Membranes were washed extensively in TTBS buffer and incubated with secondary HRP-conjugated antibodies, 10,000-fold diluted donkey anti-rabbit IgG or 20,000-fold diluted sheep anti-mouse IgG, for 1 h in TTBS buffer containing 1% bovine serum albumin. Membranes were washed (4 × 15 min) in TTBS buffer and blots were developed using ECL detection (Western Blotting Detection Reagents, Amersham Biosciences).

To estimate the level of α -toxin secretion by *S. aureus* strains, 20 µl of bacterial culture media were subjected to SDS-PAGE (15% gel) as described above. As a positive control, rHla was used at a concentration of 0.25 µg. To detect α -toxin, a 10,000-fold dilution of anti- α -hemolysin antibody (Abcam) was used, followed by incubation with 10,000-fold diluted donkey anti-rabbit IgG HRP-conjugated secondary antibodies.

Densitometric Analyses

Densitometric analyses of Western blots were performed using Kodak digital software. Results are presented as mean values of arbitrary densitometric units corrected for background intensity, or as increases over levels in nonstimulated cells.

Quantitative PCR

Total cellular RNA was extracted from cultured hMDMs using an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA samples were DNase treated and cDNA was prepared by reverse transcription using RevertAid™ First Strand cDNA synthesis kit (Fermentas, Germany). RNA (500 ng) from each sample was used for cDNA synthesis reactions with oligo (dT) primers according to the manufacturer's instructions. Quantitative PCR (qRT-PCR) reactions were performed using SYBR Green, in a reaction volume of 20 µl, containing 1 µl of cDNA sample, 0.5 µM of each primer and 1 × SYBR Green Jump-Start Taq Ready Mix. qRT-PCR forward and reverse primers for IL-6, MCL-1, and NOD2 genes, and for the housekeeping EF-2 gene (used for normalization), are listed in table 2. After 5 min of

initial denaturation at 95°C, reactions were carried out for 40 cycles in the following conditions: denaturation at 95°C for 20 s, annealing at 56–62°C (as shown in table 2) for 60 s, extension at 72°C for 60 s; followed by a final elongation step at 72°C for 10 min. All reactions were performed in duplicate. Means for threshold cycle (Ct) values were calculated and analyzed using the 'delta-delta Ct' quantification method [14]. For the evaluation of qRT-PCR reaction quality, samples were routinely resolved on nondenaturing 1.5% agarose gels and visualized by staining with ethidium bromide.

Intracytoplasmic Delivery of Staphylococcal α -Toxin Protein

Purified staphylococcal α -toxin protein (30 ng/ml) was delivered into hMDMs using the cationic lipid mixture Pro-Ject protein transfection kit. Briefly, Pro-Ject reagents were solubilized in chloroform and aliquoted into 10 μ l amounts. After chloroform evaporation, aliquots were stored at –20°C until use. α -Toxin was diluted in PBS and added to the dried Pro-Ject aliquot. After 5 min of incubation at room temperature (RT; allowing α -toxin/Pro-Ject reagent complex formation), 1 ml of RPMI 1640 medium was mixed with the sample and the whole volume was added to hMDMs previously rinsed with serum-free medium. After 3 h of incubation, cells were rinsed twice with ice-cold PBS and conditioned in serum-containing medium for an additional 5 h, until RNA extraction was performed. Efficacy of protein delivery was evaluated with the Pro-Ject FITC-labeled antibody against β -galactosidase (used as a positive control) and fluorescence microscopy analysis.

*Expression of α -Toxin mRNA by *S. aureus* inside Macrophages*

RNA was isolated from *S. aureus*-infected hMDMs using the Total RNA mini kit (A&A Biotechnology). Bacterial cells were mechanically disrupted with FastPrep-FP120 Instrument (Savant Bio101). Before cDNA synthesis, RNA was digested with RQ1 RNase (Promega) and repurified with the TRI Reagent (Ambion, Life Technologies). Next RNA (0.85 μ g) was reverse-transcribed using the cDNA High Capacity cDNA reverse transcription kit (Applied Biosystems). Transcript levels were analyzed with target specific primers, for *hla*: *hla*For-ATTTGCACCAATAAGGC CGC and *hla*Rev-TGGTTAGCCTGGCCTTCAG and for 16S rRNA: 16SFor-GCTGCCCTTTGTATTGTC and 16SRev-AGATGTTGGTTAAGTCCC. Real-time PCR was performed using a CFX96 Touch machine. Reaction mixtures consisted of 7.5 μ l of SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 1 μ l of 300 nM of primers mix, 5 μ l of 10 $\times$ diluted cDNA and 1.5 μ l water. PCR parameters were: an initial denaturation step at 94°C for 3 min, followed by 40 cycles of 10 s at 94°C, of 20 s at 55°C and of 45 s at 72°C. All samples were analyzed in triplicate. Relative transcript levels were calculated using the modified $\Delta\Delta$ Ct method [14]. PCR reactions to confirm a lack of contamination with genomic DNA were performed on RNA samples not subject to reverse transcription, using an identical protocol as for qRT-PCR.

Cytokine Assay

Cell culture (200 μ l) supernatant were collected and stored at –80°C until analyzed. The level of IL-6 was determined using a commercially available ELISA kit according to the manufacturer's instructions (R&D Systems).

Evaluation of NF κ B Activity

NF κ B activity was measured in nuclear protein extracts using the TransAM NF κ B p65 protein assay (Active Motif, Carlsbad, Calif., USA), an ELISA-based method designed to specifically detect and quantify NF κ B p65 subunit activation with high sensitivity and reproducibility. In brief, DNA binding of p65-containing NF κ B dimers was examined using a 96-well plate with an immobilized oligonucleotide containing an NF κ B consensus-binding site (5-GGGACTTTCC-3). Five micrograms of total nuclear protein was incubated in wells for 1 h at RT. These were then washed 3 times, and 100 μ l of anti-p65 subunit mAb (1:1,000 dilution) was added to each well, before incubation at RT for 1 h. Wells were again washed 3 times, and 100 μ l of HRP-conjugated secondary antibody anti-murine IgG (1:1,000 dilution) was added to each well. After 1 h at RT, plates were washed 4 times, and 100 μ l of developing solution was added to each well for 5 min. The absorbance was determined on a spectrophotometer Spectra Max Gemini EM (Molecular Devices) at 450 nm with a reference wavelength of 655 nm. TNF- α -stimulated Jurkat nuclear extracts were used as positive controls, and a 200-fold excess of wild-type (WT) and mutated NF κ B consensus oligonucleotides (all provided with the kit) were used to monitor the specificity of the assays.

Hemolysin Assay

Bacterial samples taken at the indicated time points after inoculation were standardized to the same OD (OD₆₀₀ = 1.5) and centrifuged (5,500 g at 4°C for 2 min) to remove bacteria. Supernatant (100 μ l) was diluted to 1 ml in hemolysin buffer (0.145 M NaCl, 20 mM CaCl₂) prior to the addition of 25 μ l of defibrinated rabbit blood. After incubation for 15 min at 37°C, samples were centrifuged (5,500 g at RT for 1 min) and the OD of the supernatant was measured at 543 nm. Percent hemolysis was calculated using the formula: % hemolysis = (absorbance of sample – absorbance of blank) $\times$ 100/(absorbance of positive control), where the blank is hemolysin buffer and the positive control is defibrinated rabbit blood with all erythrocytes lysed by 2% Triton X-100 in hemolysin buffer.

Statistics

Results were analyzed for statistical significance using a non-parametric Student's t test or one-way ANOVA test with the Bonferroni post hoc test. Differences were considered significant when $p < 0.05$.

Results

*Mcl-1 Induction by *S. aureus* Is Dependent on α -Toxin Expression*

Recently, we showed that *S. aureus* can protect infected macrophages from apoptosis via the upregulation of crucial antiapoptotic genes, in particular, *MCL-1* (*myeloid cell leukemia-1*). To investigate whether the induction of Mcl-1 expression in macrophages was dependent on *S. aureus* α -toxin (Hla), macrophages were infected with 3 different *S. aureus* strains: Newman, SH1000 and USA300, along with their corresponding α -toxin-

deficient mutants Δhla . The efficiency of internalization and intracellular survival for corresponding pairs (Δhla and parental WT strain) was tested 2 h after the infection of macrophages by plating cell lysates and performing CFU counts. In each case, there appeared to be little or no variation between WT and mutant strains (online suppl. fig. S1A–C; for all online suppl. material, see www.karger.com/doi/10.1159/000368048). In contrast to WT strains, however, all α -toxin-deficient mutants exhibited a significantly reduced capacity to induce *MCL-1* mRNA expression (fig. 1a). Importantly, *MCL-1* expression was determined 6 h after infection, with no statistical difference in bacterial survival within macrophages observed (fig. 1b). Collectively, these results indicate that α -toxin plays a key role in the upregulation of *MCL-1* expression in infected macrophages, in a manner independent of the ability of the toxins to abrogate killing of phagocytized *S. aureus* [7]. Interestingly, we noted that the *MCL-1* expression induced by different *S. aureus* strains does not correlate with the α -toxin levels secreted by them (fig. 1a, c). The most potent inducer of *MCL-1* expression in hMDMs is the Newman strain (fig. 1a), which secretes lower amount of toxin than USA300 (fig. 1c). Such observations indicate that α -toxin is necessary, but not sufficient, for the induction of *MCL-1* expression by *S. aureus* in infected macrophages. Since Newman appears to be the most potent inducer of *MCL-1*, and with the difference in *Mcl-1* induction compared to its Δhla mutant being significant, we routinely performed all further experiments in this strain. To verify whether the difference in induction of *MCL-1* expression changes during infection, we assessed *MCL-1* levels at different time points after macrophage challenge with bacteria. Our data revealed sustained expression of *MCL-1* following infection with the WT *S. aureus* Newman strain in comparison to its α -toxin-deficient mutant (Δhla) (fig. 2a). Further investigation revealed that similar changes also occurred at the protein level (fig. 2b), since both the Δhla mutant and heat-inactivated (HI) WT exhibited largely reduced hemolytic activity (online suppl. fig. S2), and led to markedly lower levels of *Mcl-1* expression than the parental strain. These results argue that α -toxin has an important role in the induction of *Mcl-1* expression in infected macrophages.

Mcl-1 Induction Requires the Presence of Both α -Toxin and *S. aureus*

α -Toxin, as a secretory protein, can impact the exterior of macrophages, acting at the plasma membrane or within infected cells when produced by internalized bacteria. To distinguish these two modes of action, adher-

Fig. 1. *Mcl-1* induction is dependent on α -toxin expression in various *S. aureus* strains. **a** The effect of *S. aureus* strains producing different levels of α -toxin on *MCL-1* mRNA expression in hMDMs was measured 6 h after infection by qRT-PCR. Mean values calculated from at least 3 independent experiments using hMDMs derived from different donors are shown. Bars represent mean relative expression $\pm$ SD. * $p < 0.05$. **b** hMDMs were allowed to engulf strains of *S. aureus* for 2 h at a MOI of 50, and the intracellular survival of bacteria 6 h after phagocytosis was monitored by enumeration of CFUs from cell lysates. The data are representative of at least 3 separate experiments, performed in triplicate, using hMDMs derived from different donors. Bars represent mean CFU value $\pm$ SD; n.s. = not significant. **c** Immunoblot of α -toxin level produced by *S. aureus* strains. A representative immunoblot from 3 separate experiments is shown.

Fig. 2. Mcl-1 expression at both mRNA and protein levels depends on α -toxin expression in the *S. aureus* Newman strain. **a** Time-course of *MCL-1* expression following *S. aureus* (WT and Δhla mutant) infection, measured at different time points between 2 and 48 h after infection using qRT-PCR. Bars represent mean relative expression $\pm$ SD; * $p < 0.05$. n.s. = Not significant. **b** The effect of phagocytosis on the WT Newman strain, Δhla mutant (Δhla) and inactivated, heat-killed *S. aureus* (HI) on Mcl-1 protein synthesis measured in hMDMs 6 h after infection by immunoblot. A representative immunoblot from 3 separate experiments is shown.

ent macrophages were treated with purified α -toxin or else it was injected directly into the cells using an intracytoplasmic delivery system. Surprisingly, neither the extracellular nor intracellular application of α -toxin alone had any effect on *MCL-1* expression, as determined by q-PCR and immunoblot analysis (fig. 3a, upper and lower panels, respectively). In contrast to macrophages infected with live, toxin-producing *S. aureus*, which express a large amount of Mcl-1, the Mcl-1 levels in toxin-treated macrophages remained at the same low level as mock-infected cells (fig. 3a). These results indicated that α -toxin alone does not affect Mcl-1 expression per se, but is perhaps required to enhance Mcl-1 expression induced by *S. aureus*. To investigate this possibility, macrophages infected with the *S. aureus* Δhla

strain were treated with purified α -toxin, and the effects on *MCL-1* expression were assessed. As shown in figure 3b, the extracellular application of purified recombinant α -toxin resulted in its internalization by macrophages (fig. 3c) and potent upregulation of *MCL-1* expression in macrophages infected with Newman Δhla , to the levels observed upon infection with the WT strain. The role of α -toxin in *MCL-1* induction observed in hMDMs infected with the WT strain is further supported by sustained *hla* mRNA levels by intracellular *S. aureus* cells (fig. 3d). These results confirm that intracellularly distributed α -toxin in the context of live bacteria is a prerequisite for full-scale induction of Mcl-1 expression in *S. aureus*-infected macrophages.

α -Toxin-Dependent Mcl-1 Induction Is Regulated by IL-6

We have recently shown that IL-6 plays a role in *S. aureus*-mediated Mcl-1 expression in hMDMs [13]. To determine the role of α -toxin in this process, macrophages were stimulated with Newman or its Δhla mutant strain. Our results revealed that the mutant exhibited a significantly reduced capacity to induce IL-6 secretion by hMDMs (fig. 4a). Moreover, we observed that complementation of the mutant with purified α -toxin, or bacterial growth medium containing active toxin (as determined by hemolysis assay; see online suppl. fig. S2), resulted in significantly higher levels of IL-6 secretion (fig. 4a), in a manner similar to the enhancement of *MCL-1* expression illustrated in figure 3b. Thus, α -toxin contributes to enhanced IL-6 expression as well as the upregulation of *MCL-1*. The importance of IL-6 in the upregulation of Mcl-1 mediated by α -toxin-exposed hMDMs was verified by treatment of hMDMs with the IL-6R neutralizing antibodies. As shown in figure 4b, anti-IL-6R antibodies dampened *MCL-1* expression induced by the toxin-producing Newman strain; however, the antibodies had no significant effect on cells infected with the Δhla mutant. Together, these results demonstrate that α -toxin-induced *MCL-1* expression is mediated by IL-6.

The Role of Extracellular TLR-Dependent Signaling in *S. aureus*-Dependent Mcl-1 Upregulation

The activation of signaling pathways in response to bacterial infection is mediated by a broad range of pattern-recognition receptors. Amongst such extracellular receptors, *S. aureus* interacts mostly with TLR2. As such, the participation of TLR2 and TLR4 in the induction of Mcl-1 expression by *S. aureus* was assessed using anti-TLR2 and anti-TLR4 neutralizing antibodies. Pretreat-

Fig. 3. The indirect effect of α -toxin on Mcl-1 expression. **a** The effect of extracellular (external rHla) or intracellular (internal rHla) application of recombinant *S. aureus* α -toxin (30 ng/ml) on Mcl-1 expression in hMDMs was compared to infection with live *S. aureus* (WT Newman strain). Upper panel: the relative amount of *MCL-1* mRNA 6 h after treatment with α -toxin was measured by qRT-PCR. Mean values calculated from at least 3 independent experiments using hMDMs derived from different donors are shown. Bars represent mean relative expression $\pm$ SD; ** $p < 0.01$. n.s. = Not significant. Lower panel: Mcl-1 protein synthesis measured in hMDMs 20 h after infection by immunoblot. A representative immunoblot from 3 separate experiments is shown. **b** *MCL-1* upregulation is restored for α -toxin-deficient strains (Δhla) by the addition of rHla. hMDMs were infected with *S. aureus* strains at an MOI of 1:50, with or without coadministration of α -toxin (30

ng/ml). RNA was isolated 8 h after infection and *MCL-1* expression was determined by qRT-PCR. Mean values calculated from at least 3 independent experiments using hMDMs derived from different donors are shown. Bars represent mean relative expression $\pm$ SD; * $p < 0.05$. **c** The presence of α -toxin (rHla) within macrophages visualized by immunoblot. hMDMs were infected with *S. aureus* strains at an MOI of 1:50, with or without coadministration of α -toxin (30 ng/ml). Protein was isolated 8 h after infection and the presence of α -toxin was determined by immunoblot. A representative immunoblot from 2 separate experiments using hMDMs derived from different donors is shown. **d** Relative α -toxin (*Hla*) mRNA expression by Newman WT inside hMDMs up to 48 h after infection, normalized to CFUs recovered from the intracellular compartment. These representative results were obtained from 2 independent experiments.

ment of hMDMs with anti-TLR2 antibodies only partially inhibited ($31.5 \pm 10.5\%$ inhibition) the Mcl-1 expression induced by *S. aureus* whereas anti-TLR4 antibodies had no effect (fig. 5a). Similar results were obtained for IL-6 secretion, which was marginally inhibited only by TLR2-neutralizing antibodies (fig. 5b). The partial effect of blocking TLR2-mediated signaling pathways on both IL-6 and Mcl-1 expression suggests the involvement of alternative receptors in *S. aureus* recognition.

Intracellular Recognition of S. aureus Plays a Role in α -Toxin-Dependent Mcl-1 Expression

α -Toxin facilitates escape of the pathogen to the cytoplasm, thus ensuring the access of bacterial cell wall components to the cytoplasmic milieu [7]. Therefore, the in-

volvement of an alternative intracellular recognition receptor would explain the lack of significant inhibition of Mcl-1 and IL-6 expression by blocking extracellular TLR-dependent signaling pathways (fig. 5a, b). Since activation of the NF κ B transcription factor is a crucial step in the regulation of cell survival, which can regulate both Mcl-1 and IL-6 expression, we therefore determined the effect on NF κ B activity of inhibiting bacterial engulfment. As expected, bacterial internalization was significantly inhibited by treating cells with cytochalasin D (5 μ M; fig. 6a). Interestingly, NF κ B activity induced by toxin-producing *S. aureus* was also significantly inhibited by cytochalasin D, yet there was no effect on NF κ B activation induced by the Δhla mutant (fig. 6b). This observation was confirmed by the *MCL-1* expression profile

Fig. 4. α -Toxin-induced Mcl-1 expression is mediated by IL-6. **a** hMDMs were incubated for 6 and 24 h with either the WT Newman strain or Δhla mutant at a MOI of 50, in the presence or absence of α -toxin (rHla, 30 ng/ml), or conditioned media (0.3% v/v). IL-6 levels in supernatants were determined by ELISA. Data represent the mean $\pm$ SD of 3 independent experiments; * $p < 0.05$; ** $p < 0.01$. **b** hMDMs were preincubated with anti-IL-6R antibodies

(1 μ g/ml) for 30 min, and then infected with the indicated *S. aureus* strains (WT or Δhla) at a MOI of 1:50 or stimulated with IL-6 (200 ng/ml). At 7 h after infection, RNA was extracted and relative *MCL-1* expression was measured using qRT-PCR. Mean values calculated from at least 3 independent experiments using hMDMs derived from different donors are shown. Bars represent mean relative expression $\pm$ SD. * $p < 0.05$; n.s. = not significant.

Fig. 5. The role of TLR2 in *S. aureus*-induced Mcl-1 expression. hMDMs were preincubated with anti-TLR2 (5 μ g/ml) or anti-TLR4 (5 μ g/ml) antibodies for 30 min, and then infected with *S. aureus* Newman at a MOI of 1:50. **a** The effect of *S. aureus* on Mcl-1 protein synthesis was measured 20 h after infection by immunoblot. A representative immunoblot from 3 independent ex-

periments performed with hMDMs derived from different donors is shown. **b** The effect of *S. aureus* (WT) on IL-6 secretion 20 h after infection. IL-6 concentration in conditioned media from hMDMs derived from 2 different donors was determined by ELISA. Data represent mean values $\pm$ SD.

(fig. 6c) and secretion of TNF- α and IL-6 cytokines, which are regulated by NF κ B (fig. 6d, e). These results confirm the role of α -toxin in amplifying antiapoptotic signaling pathways by *S. aureus* through exposure of antigens to intracellular receptors other than extracellular TLR.

α -Toxin Contributes Significantly to the Inhibition of Macrophage Apoptosis

Once we had demonstrated that α -toxin potentiated the Mcl-1 expression induced by *S. aureus*, we set out to investigate the role of α -toxin in cytoprotection mediated by

Fig. 6. NFκB activation, *MCL-1* expression and cytokine secretion induced by *S. aureus* are dependent on α-toxin expression. Human macrophages were preincubated with cytochalasin D (cytD; 5 μM) for 30 min and then infected with *S. aureus* (Newman WT or Δ*hla* mutant) at a MOI of 50. **a** The effect of cytochalasin D treatment on internalization of bacteria by macrophages. The intracellular survival of bacteria 2 h after phagocytosis was monitored by enumeration of CFUs from cell lysates. The data shown are representative of at least 3 separate experiments, performed in triplicate, using hMDMs derived from different donors. Bars represent mean CFU value ± SD; * *p* < 0.05; ** *p* < 0.01. **b** The activity of NFκB in

macrophages was determined 1 h after infection by ELISA. **c** The relative amount of *MCL-1* mRNA 6 h after treatment with α-toxin was measured by qRT-PCR. Mean values calculated from 3 independent experiments using hMDMs derived from different donors are shown. Bars represent mean relative expression ± SD; * *p* < 0.05; *** *p* < 0.001. n.s. = Not significant. TNF-α (**d**) and IL-6 (**e**) secretion by macrophages 20 h after infection determined by ELISA. Data are representative of 3 independent experiments performed in duplicate using hMDMs derived from different donors. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

S. aureus in more detail. Accordingly, murine macrophages (RAW 264.7 cells) were separately infected with either the Newman parent or its Δ*hla* mutant, followed by induction of apoptosis with staurosporin. As described previously [8], bacterial PGN induces intracellular signaling and subsequent cytokine secretion, so *S. aureus* PGN was used as an additional control. At 2 h after infection (or treatment with 2 μg/ml of PGN), apoptosis was induced with staurosporin. All tested conditions resulted in a 2- to 6-fold inhibition of caspase-3 activity induced by staurosporin (fig. 7). Importantly, the Newman strain induced significantly

stronger inhibition than the Δ*hla* mutant, while PGN yielded the weakest effect. Additional treatment of RAW 264.7 cells with conditioned media collected from the WT (α-toxin-positive) strain resulted in enhanced cytoprotection in cells infected with either of the two bacterial strains as well as those treated with PGN (fig. 7). Together, these results demonstrate that complementation of bacterial infection with α-toxin leads to a stronger inhibition of apoptosis in staurosporin-treated macrophages. These results are consistent with our Mcl-1 expression data (fig. 3b) and confirm the protective role of Mcl-1, mediated by α-toxin.

Fig. 7. Cytoprotection of *S. aureus*-infected macrophages is dependent on α -toxin. The enhancement of *S. aureus* (Newman WT and Δhla mutant) and/or PGN-mediated caspase-3 inhibition by bacterial-conditioned media was measured using DEVD-AFC as a substrate. RAW 264.7 cells were infected with *S. aureus* (MOI 1:5) or treated with PGN (2 μ g/ml) for 2 h, with or without bacterial growth media (0.3% v/v) added, followed by stimulation for 6 h with STS. Bars represent mean $\pm$ SD of caspase-3 activity (RFU/min) from 3 independent experiments. The caspase-3 activity of mock-infected cells treated with STS was set as 100%. * $p < 0.05$; ** $p < 0.01$.

Discussion

Staphylococcus aureus invades macrophages and promotes cytoprotection of infected cells, thus creating a safe niche for silent persistence. This process occurs through the upregulation of crucial antiapoptotic genes and, particularly, *MCL-1*. The increase of Mcl-1 expression, which occurs shortly after infection and is sustained at high levels long after the engulfment of live *S. aureus*, is in stark contrast to the effects induced by *Escherichia coli*, heat-killed staphylococci or latex beads. This strongly argues that Mcl-1 expression is specifically induced and maintained by factors released by viable intracellular staphylococci [13]. Furthermore, only α -toxin-positive *S. aureus* strains are able to survive phagocytosis by macrophages beyond 24 h after infection [7], suggesting that this toxin may function as an effector molecule. Consistent with this idea, strains that possess high α -toxin levels induce significantly higher levels of Mcl-1, and consequently exert stronger cytoprotective effects. Conversely, our data reveal that α -toxin does not act alone, but rather

in conjunction with other bacterial antigens, e.g. PGN. Thus, the effects of α -toxin are indirect, and promote escape of bacteria from the phagosomes into the cytosol (fig. 7), akin to that recently reported for professional phagocytes [7]. This process of releasing *S. aureus* from the endosomes of epithelial cells is also complemented by other pathogen-derived toxins, such as δ -toxin and β -toxin [6, 15]. This facilitates the recognition of PAMPs (pathogen-associated molecular patterns) from *S. aureus* by intracellular receptors. This hypothesis is strongly supported by results showing that the inhibition of extracellular TLRs does not ablate Mcl-1 induction, which argues for additional and alternative intracellular PAMP sensing. Such a candidate molecule is NOD2, since its abundance is dramatically increased in infected macrophages (online suppl. table S1; qRT-PCR data from 6 independent blood donors revealed 3.6- to 11.3-fold increases above mock-infected cells), suggesting that NOD2 might be involved in sensing the presence of intracellular *S. aureus*. This would be in agreement with recent findings by Hruz et al. [8], showing that NOD2-dependent recognition of *S. aureus* and muramyl dipeptide is facilitated by α -toxin; and is associated with the induction of cytokine release, including IL-6 and IL-8. In this context, the finding that IL-6 secretion by macrophages was enhanced by *S. aureus* in the presence of α -toxin lends further support to the idea that NOD2 is exploited by intracellular *S. aureus*. This leads to increased levels of Mcl-1, and subsequent cytoprotection through an as yet unknown intracellular receptor (fig. 8). Work is currently underway in our laboratory to specifically determine the identity of this protein.

Such a hypothesis is in agreement with the observation that the autocrine role of IL-6 has previously been shown to be essential for *S. aureus*-induced Mcl-1 expression, and for cytoprotection in infected macrophages (fig. 8) [13]. As such, the fact that cytochalasin D had differing effects on NF κ B activation and *MCL-1* expression induced by α -toxin-producing and deficient strains, is perhaps the most compelling result in favor of a role for intracellular receptors in *S. aureus*-mediated signaling. This is supported by the observation that IL-6 regulation in infected macrophages is at least partly mediated by NF κ B activation. As expected, blocking phagocytosis of the toxin-null mutant did not affect signaling via extracellular TLRs, and subsequent NF κ B activation. Conversely, the same treatment significantly reduced NF κ B activation by toxin-positive strains, presumably because it prevented pathogen recognition by intracellular PAMP receptors. These findings clearly indicate that extracel-

Fig. 8. Schematic representation for the role of α -toxin in the inhibition of macrophage apoptosis by *S. aureus*. *S. aureus* is recognized by TLR2 (1), initiating a signaling cascade via the NF κ B pathway (2). Simultaneously, phagocytosed bacteria (3) escape from phagosomes through α -toxin-mediated lysis of the membrane (4). The cytoplasmic bacteria then interact with intracellular/cytoplasmic receptors (5), triggering signaling pathways that lead to NF κ B activation (6). Activated NF κ B either directly (7), or indirectly, via IL-6-dependent autocrine signaling (8), stimulates Mcl-1 expression, which results in cytoprotection of the infected cells.

lular TLRs play a major role in signal transduction pathways engaged by α -toxin-deficient staphylococcal strains, since intracellular receptors would be inaccessible to them.

Work by others has shown that α -toxin alone is sufficient to induce cell death in host cells [16], with higher concentrations predominantly producing necrosis, and low doses resulting in apoptosis [17]. Notably, in our studies, neither culture supernatants nor purified α -toxin at any of the concentrations tested affected hMDM viability (online suppl. fig. S3). These results mirror the cellular response to live *S. aureus*. While in some cells, *S. aureus* induces rapid cell death [18, 19], in macrophages, intracellular bacteria ensure protection of the infected cell against death [12]. Thus, we present a novel mechanism for the cellular response to *S. aureus*, facili-

tated by α -toxin located in intracellular niches that is specific to macrophages. This model is compatible with reports that α -toxin can facilitate membrane repair, not only through activation of the central regulators of membrane biogenesis (sterol regulatory element-binding proteins), but also by modulating p38 and EGF signaling [20–22]. Furthermore, the discovery of ADAM10 as a high-affinity α -toxin receptor confirms this novel role of α -toxin as a cell signaling modulator [23]. Indeed, the extracellular activity of α -toxin was also noted in our study, since the response of cytochalasin D-treated macrophages to WT and α -toxin-deficient strains, as monitored by NF κ B activity and IL-6 expression, was dramatically different (fig. 6b, e).

Our findings present a new face for α -toxin as a potent virulence factor. When expressed by *S. aureus* inside macrophages, the toxin facilitates the escape of bacteria into the cytoplasm [7], modifies the host immune response and promotes the cytoprotection of infected macrophages (fig. 8). Consequently, this enables these long-lived, mobile immune cells to migrate away from the primary site of colonization, and facilitate dissemination of the pathogen. Additional *in vivo* studies are necessary to confirm whether this manipulation of macrophage viability contributes primarily to pathogen dissemination or is, conversely, actually beneficial to the host.

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