α-Toxin is a mediator of *Staphylococcus aureus*–induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling

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Infections with *Staphylococcus aureus*, a common inducer of septic and toxic shock, often result in tissue damage and death of various cell types. Although *S. aureus* was suggested to induce apoptosis, the underlying signal transduction pathways remained elusive. We show that caspase activation and DNA fragmentation were induced not only when Jurkat T cells were infected with intact bacteria, but also after treatment with supernatants of various *S. aureus* strains. We also demonstrate that *S. aureus*–induced cell death and caspase activation were mediated by α-toxin, a major cytotoxin of *S. aureus*, since both events were abrogated by two different anti–α-toxin antibodies and could not be induced with supernatants of an α-toxin–

deficient *S. aureus* strain. Furthermore, α-toxin–induced caspase activation in CD95-resistant Jurkat sublines lacking CD95, Fas-activated death domain, or caspase-8 but not in cells stably expressing the antiapoptotic protein Bcl-2. Together with our finding that α-toxin induces cytochrome c release in intact cells and, interestingly, also from isolated mitochondria in a Bcl-2–controlled manner, our results demonstrate that *S. aureus* α-toxin triggers caspase activation via the intrinsic death pathway independently of death receptors. Hence, our findings clearly define a signaling pathway used in *S. aureus*–induced cytotoxicity and may provide a molecular rationale for future therapeutic interventions in bacterial infections.

Introduction

Apoptosis, or programmed cell death, is a genetically well-defined intrinsic suicide program that enables an organism to remove mutated or infected cells without causing an inflammatory reaction. Cells undergoing apoptosis show a characteristic sequence of morphological and biochemical features including membrane blebbing, cellular shrinkage, and condensation of chromatin. Recent studies demonstrate that these key morphological alterations of apoptosis are mediated essentially by a family of intracellular cysteine proteases called caspases (Cohen, 1997; Cryns and Yuan, 1998; Los et al., 1999).

Caspases are synthesized as inactive proenzymes and proteolytically processed to form an active complex composed of two heterodimeric subunits of ∼10 and 20 kD. Based on

their structure and order in cell death pathways, caspases can be divided into initiator and effector caspases. Initiator caspases, such as caspase-8 or -9, exert regulatory roles. Upon binding to signal transducing molecules, they activate downstream effector caspases such as caspase-3, -6, or -7, which finally cleave various cellular substrates, thereby inducing the demise of the cell (Cryns and Yuan, 1998; Stroh and Schulze-Osthoff, 1998; Porter and Jänicke, 1999).

Activation of caspases is achieved via two principal signaling pathways, namely the extrinsic and intrinsic death pathways (for review see Schulze-Osthoff et al., 1998; Li and Yuan, 1999). The extrinsic death pathway involves the ligation of death receptors (CD95/Fas/APO-1; TNF receptor-1) that leads to the recruitment of the adapter molecule Fas-activated death domain (FADD)* and pro–caspase-8 into a

*Abbreviations used in this paper: AMC, aminomethyl coumarin; DEVD, *N*-acetyl-Asp-Glu-Val-Asp; FADD, Fas-activated death domain; IETD, *N*-acetyl-Ile-Glu-Thr-Asp; LEHD, *N*-acetyl-Leu-Glu-His-Asp; PBL, peripheral blood lymphocyte.

death-inducing signaling complex. The intrinsic death pathway is initiated at the mitochondrion by the release of cytochrome c, a process that can be inhibited by antiapoptotic proteins of the Bcl-2 family. When released, cytosolic cytochrome c binds together with dATP and the apoptosis-activating factor-1 to pro–caspase-9 to form the apoptosome. Upon formation of the death-inducing signaling complex or the apoptosome, pro–caspase-8 or -9, respectively, are autoproteolytically processed, resulting in the activation of downstream caspases.

Numerous death stimuli can trigger apoptosis, and even several species of microbial pathogens were shown in recent years to activate and modulate components of the cellular death machinery (Gao and Kwaik, 2000). Amongst these belongs *Staphylococcus aureus*, one of the most common gram-positive bacterial pathogens in humans that plays an increasing role in nosocomial infections (Lowy, 1998). Breached mucocutaneous membranes or impaired host immunity facilitate tissue invasion and bloodstream dissemination of *S. aureus*. This is followed by serious infections, such as abscess formation, osteomyelitis, endocarditis, or pneumonia, which often require a prolonged and aggressive antibiotic treatment. Among the most serious complications of *S. aureus* infections are manifestations of septic and toxic shock syndromes that may lead to multiple organ failure (Marrack and Kappler, 1990).

Since tissue injury and a depletion of immune cells, including macrophages and T cells, are characteristic features of septic and toxic shock syndromes, several studies in the past decade have focused on cell death induction after exposure to microbial pathogens (for review see Weinrauch and Zychlinsky, 1999; Gao and Kwaik, 2000). In contrast to its original function to eliminate cells without causing an inflammatory reaction, there is growing evidence that apoptotic tissue damage or immune suppression may contribute significantly to the risk of secondary opportunistic infections (Oberholzer et al., 2001). A paradigm of bacteria-induced apoptosis is the infection of macrophages with *Shigella flexneri*, which involves the activation of caspase-1 and release of IL-1β, resulting in an acute inflammatory response (Zychlinsky et al., 1992). On the other hand, *Yersinia enterocolitica* translocates soluble proteins belonging to the Yop family in the host that can inhibit the activation of the antiapoptotic transcription factor NF-κB (Ruckdeschel et al., 1998). It has also been shown that some bacteria such as *Pseudomonas aeruginosa* or *Helicobacter pylori* utilize the CD95 death receptor/ligand system to induce apoptosis in infected target cells (Rudi et al., 1998; Grassme et al., 2000). Nevertheless, although pathogens possess a plethora of strategies to control the fate of the host cell, in most cases the underlying mechanisms of bacteria-induced cell death remain unclear.

In the present study, we investigated the mechanism of cell death in T cells after *S. aureus* infection. We show that not only in Jurkat T-lymphocytes but also in human peripheral blood lymphocytes (PBLs) and monocytes *S. aureus*–induced cytotoxicity can be mediated by α-toxin, a soluble pore-forming toxin, and does not require bacterial internalization. We further demonstrate that α-toxin–induced cytotoxicity and caspase activation do neither involve CD95 nor other death receptors but instead the activation of the mito-

chondrial apoptosis machinery. Interestingly, α-toxin was able to induce the proapoptotic release of cytochrome c not only from intact cells but also from isolated mitochondria. Therefore, our results clearly define a signaling pathway of *S. aureus*–induced cell death and indicate that besides pore formation in the plasma membrane, *S. aureus* α-toxin utilizes a direct mechanism to trigger cell death in target cells.

Results

A soluble factor in supernatants of *S. aureus* cultures is sufficient for cell death induction

To assess the cytotoxic potential of different *S. aureus* strains and investigate whether intact bacteria are required for this process, Jurkat leukemic T cells were incubated with various cytotoxic or noncytotoxic *S. aureus* strains or with their respective culture supernatants. As measured by the formation of hypodiploid DNA, both washed whole bacteria (Fig. 1 A) of the cytotoxic and hemolytic *S. aureus* strains 6850 and RN6390 and their respective supernatants (Fig. 1 B) efficiently induced apoptosis of Jurkat cells in a dose- and time-dependent manner. Remarkably, cell death induction by either supernatants or intact bacteria was achieved to a similar extent and with similar kinetics as with an agonistic anti-CD95 antibody. In contrast, the noncytotoxic and nonhemolytic *S. aureus* strain Cowan I and the nonpathogenic *S. carnosus* strain TM300 did not induce cell death.

α-Toxin is a mediator of *S. aureus*–induced cell death

We next investigated the nature of the soluble factor in *S. aureus* culture supernatants, which was responsible for cell death induction. Interestingly, in Coomassie-stained SDS-polyacrylamide gels, we observed a remarkable correlation between the expression levels of α-toxin, the major cytolysin of *S. aureus*, and the cytotoxic potential of the various *S. au-*
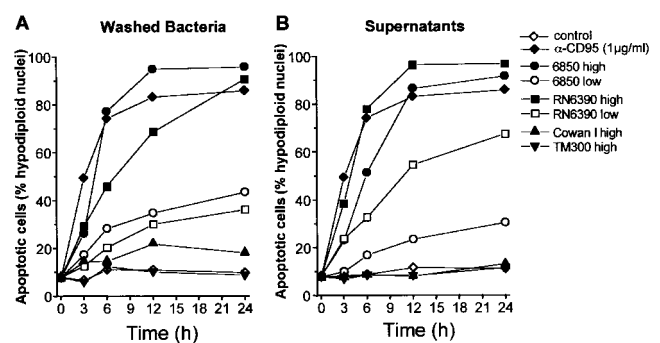

Figure 1. **Both intact *S. aureus* cells and bacterial supernatants induce T cell apoptosis.** Jurkat cells were incubated with live washed bacteria (A) or sterile-filtered supernatants of the same bacterial cultures (B). After the indicated times, the proportion of apoptotic cells was determined by flow cytometry. (A) Fresh suspensions of the indicated bacterial strains were added to Jurkat cells, resulting in a MOI of 30 (low) and 120 (high). Cells were incubated on ice for 2 h to allow sedimentation and then shifted to 37°C. Lysostaphin (20 μg/ml) was added to lyse and kill *staphylococci*. Lysostaphin without bacteria served as a negative control, whereas agonistic anti-CD95 was used as a positive control. (B) Fresh bacterial supernatants were added to Jurkat cells, resulting in a final concentration (vol/vol) of 0.1% (low) and 1% (high).

reus strains (unpublished data). *S. aureus* α-toxin has been reported to damage cells by the generation of pores in the plasma membrane (Jonas et al., 1994); however, its precise role in apoptotic pathways has not been established yet. To analyse the cytotoxic potential of this molecule in more detail, Jurkat T cells were incubated with various concentrations of a commercially available α-toxin in the absence or presence of an α-toxin neutralizing antibody. As shown in Fig. 2 A, ∼67% of Jurkat cells were apoptotic after treatment with α-toxin concentrations ranging from 0.1 to 10 μg/ml. The addition of the α-toxin neutralizing antibody resulted in a dose-dependent inhibition of cell death

Figure 2.   ***S. aureus* α-toxin is required for induction of apoptosis.** (A) Effect of purified α-toxin. Jurkat cells were preincubated for 30 min with various dilutions of a sheep anti–α-toxin antiserum and then incubated with the indicated concentrations of the commercially available preparation of α-toxin. (B and C) Anti–α-toxin neutralizes the proapoptotic activity of *S. aureus* supernatants. Jurkat cells were preincubated with various dilutions of a sheep anti–α-toxin antiserum, a rabbit anti–α-toxin antiserum, or normal rabbit serum (NRS) for 30 min. Subsequently, sterile-filtered supernatants of RN6390 (B) or Wood 46 (C) were added at the indicated dilutions, and formation of hypodiploid nuclei was assessed. (D) α-Toxin–deficient *S. aureus* does not induce apoptosis. Jurkat cells were incubated with various concentrations of supernatants of the α-toxin–producing strain DU5883 or its α-toxin–deficient counterpart DU1090. (E) Coomassie-stained SDS-PAGE of a highly purified (lane 2) and the commercially available (lane 3) α-toxin preparation. The molecular sizes of the protein marker used in lane 1 are indicated on the left. (F) Jurkat cells were incubated with the indicated concentrations of the two α-toxin preparations, and cell death was assessed after 24 h.

achieved with 0.1 or 1 μg/ml α-toxin. In contrast, the antibody could not inhibit cell death when 10 μg/ml α-toxin were used (Fig. 2 A) or when apoptosis was induced with anti-CD95 (unpublished data), demonstrating the specificity of the α-toxin antibody. More interestingly, when Jurkat cells were treated with supernatants of the moderately and highly cytotoxic *S. aureus* strains RN6390 (Fig. 2 B) and Wood 46 (Fig. 2 C), respectively, apoptosis could be inhibited completely with two different neutralizing α-toxin antibodies. Normal rabbit serum that was used as a control had no effect. To further verify this finding, we incubated Jurkat cells with supernatants of the α-toxin–producing but FnBP-deficient and thus noninvasive *S. aureus* strain DU5883 and DU1090, an α-toxin knock-out mutant. Only the supernatant of the α-toxin–producing strain DU5883 was able to induce the dose-dependent death of Jurkat cells, whereas the supernatant of the α-toxin knock-out strain DU1090 had no effect (Fig. 2 D).

SDS-polyacrylamide electrophoresis revealed minor impurities of the commercially available α-toxin preparation (Fig. 2 E, lane 3). To exclude that these contaminants contributed to the observed cell death induction, we compared the cytotoxic effect of this preparation with highly purified α-toxin (Palmer et al., 1993), which is characterized as a single band with the expected molecular weight of 34 kD (Fig. 2 E, lane 2). As both α-toxin preparations efficiently induced death of Jurkat cells with a similar dose dependency (Fig. 2 F), our data clearly demonstrate that α-toxin is a soluble mediator of *S. aureus*–induced cell death.

α-Toxin is required for *S. aureus*–induced caspase-3 activation

Apoptosis is mediated essentially by activation of the caspase cascade, resulting in cleavage of several death substrates. To examine the cell death pathway induced by *S. aureus* and especially by α-toxin in more detail, we incubated Jurkat cells with supernatants of the various *S. aureus* strains and analyzed the cell extracts for caspase-3 activity as determined by their ability to cleave the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-aminomethyl-coumarin (DEVD-AMC). Supernatants of the *S. aureus* strains Wood 46, RN6390, 6850, and DU5883 significantly induced caspase-3 activity in a dose-dependent manner, whereas no DEVD-AMC cleavage activity could be detected in untreated Jurkat cells or in cells incubated with supernatants of the nontoxic *S. carnosus* strain TM300 (Fig. 3 A). DEVDase activity was first detected 75 min after stimulation and reached a plateau at ∼3 h (unpublished data). Interestingly, the supernatants of the cytotoxic *S. aureus* strains induced a sharp increase in caspase-3 activity which, however, declined rapidly after an individual peak was reached. It is also noteworthy that the extent of caspase-3 activation correlated directly with the cytotoxic potential of the various *S. aureus* strains and was most pronounced with supernatants of Wood 46 and RN6390 (Fig. 2, B and C, compared with Fig. 3 A).

To demonstrate that *S. aureus*–induced caspase-3 activation was mediated by α-toxin, we also measured DEVD-AMC cleavage activity in cell extracts of Jurkat cells treated with α-toxin or with Wood 46 supernatant in the presence of the neutralizing anti–α-toxin antibody. As expected, the
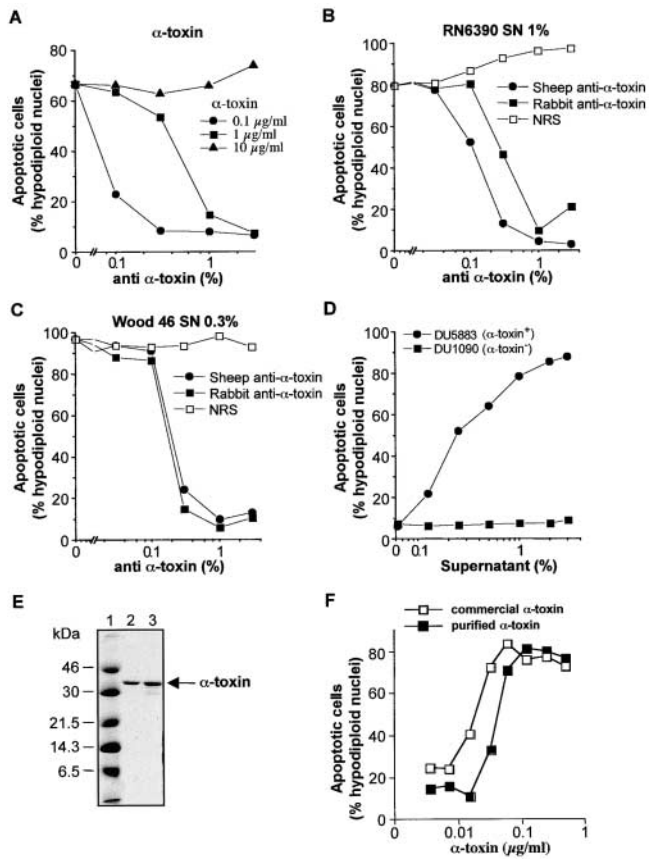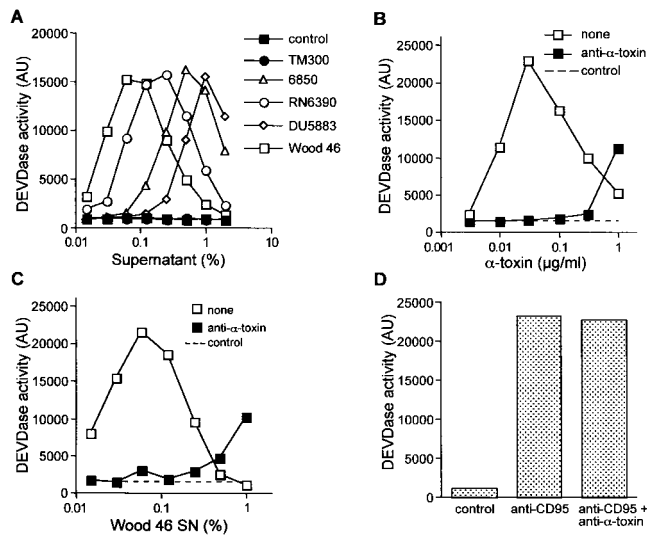
Figure 3. **S. aureus–induced caspase-3 activation is mediated by α-toxin.** (A) DEVDase activity in extracts of Jurkat cells incubated for 4 h with the indicated supernatant dilutions of the various S. aureus strains. (B–D) DEVDase activity in extracts of Jurkat cells incubated for 4 h with the indicated concentrations of α-toxin (B), Wood 46 supernatant (SN) (C), or the agonistic anti-CD95 antibody (D) in the absence or presence of the neutralizing sheep anti–α-toxin antiserum (1:100). The dashed line (control) represents the DEVDase activity in untreated cells.

Figure 4. **S. aureus–induced caspase-3 activation results in α-fodrin cleavage and DNA fragmentation.** (A) Western blot analyses demonstrating caspase-3 processing (top) and α-fodrin cleavage (bottom) in Jurkat cells that were either left untreated (control) or incubated for 4 h with the indicated dilutions of Wood 46 supernatant (SN). (B) Agarose gel electrophoretic separation of DNA extracted from Jurkat cells that were either left untreated (control) or incubated for 6 h with the indicated dilutions of Wood 46 supernatant (SN). The positions of the DNA molecular size marker are indicated on the right.

antibody completely blocked α-toxin– and Wood 46–induced caspase-3 activation (Fig. 3, B and C, respectively). On the other hand, activation of caspase-3 induced by the agonistic anti-CD95 antibody was not affected, demonstrating again the specificity of the anti–α-toxin antibody (Fig. 3 D). Consistent with our results shown in Fig. 2 A, the antibody could also not inhibit caspase-3 activation when higher (>1 μg/ml) α-toxin concentrations were used (Fig. 3 B). Similar results were obtained with supernatant of the S. aureus strain Wood 46 (Fig. 3 C) and the highly purified α-toxin, which induced caspase-3 activation in these cells with an almost identical dose dependency as the commercially available preparation (unpublished data).

Western blot analysis confirmed these results, showing that treatment of Jurkat cells with supernatant of Wood 46 resulted in the rapid conversion of the inactive 32 kD caspase-3 precursor into the proteolytically active p17 subunit, indicating that caspase-3 was indeed activated during S. aureus–induced apoptosis (Fig. 4 A, top). Similar results were obtained when α-toxin was used as the death stimulus (unpublished data). In both cases, the appearance of the active p17 caspase-3 subunit was abrogated completely in the presence of the anti–α-toxin antibody (unpublished data), demonstrating that caspase-3 activation was induced by α-toxin. In agreement with the DEVDase assays (Figs. 3, A and C), caspase-3 activation peaked at lower Wood 46 concentrations (0.06% supernatant) and declined rapidly when higher concentrations were used (Fig. 4 A, top). Furthermore, caspase-3 activation correlated directly with cleavage of the death substrate α-fodrin into the 120-kD fragment (Fig. 4 A, bottom) and the appearance of the characteristic DNA ladder formation (Fig. 4 B), both of which could be inhib-
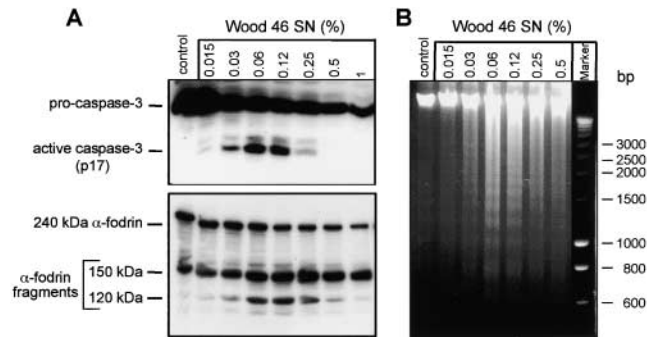
ited completely by the anti–α-toxin antibody (unpublished data). Since it was shown recently that active caspase-3 is absolutely required for the induction of both these events (Jänicke et al., 1998a,b), our results clearly demonstrate that S. aureus–induced cell death and caspase activation are mediated by α-toxin.

α-Toxin induces caspase-3 activation in lymphocytes and monocytes

Infection with S. aureus is accompanied by the death of immune competent cells including T cells and monocytes (Weinrauch and Zychlinsky, 1999; Gao and Kwaik, 2000). To analyze the role of α-toxin in this respect and to establish a more general mechanism on various clinically relevant cell types, human PBLs and monocytes were incubated for the indicated times with various concentrations of the two α-toxin preparations. As shown in Fig. 5, caspase-3 was activated efficiently in PBL and monocytes by both α-toxin preparations in a time- and dose-dependent manner. Similar to the results obtained with Jurkat cells (Fig. 2 F; unpublished data), both α-toxin preparations induced activation of caspase-3 in PBL or monocytes with almost identical efficiencies, indicating that α-toxin might contribute to cell death observed after S. aureus infection.

α-Toxin activates the initiator caspases 8 and 9

Caspase-3 activation can be achieved either by caspase-8, the most proximal caspase in death receptor signaling, or by caspase-9, the initiator caspase of the mitochondrial pathway. To elucidate which of these two pathways is responsible for α-toxin–induced caspase-3 activation, extracts of Wood 46–treated Jurkat cells were analyzed for their capability to cleave the fluorogenic substrates N-acetyl-Ile-Glu-Thr-Asp (IETD)-AMC and N-acetyl-Leu-Glu-His-Asp (LEHD)-AMC, which are indicative of caspase-8 and -9 activity, respectively. In addition, Western blot analyses were performed. Similar to caspase-3 activation (Fig. 3), Wood 46 induced a
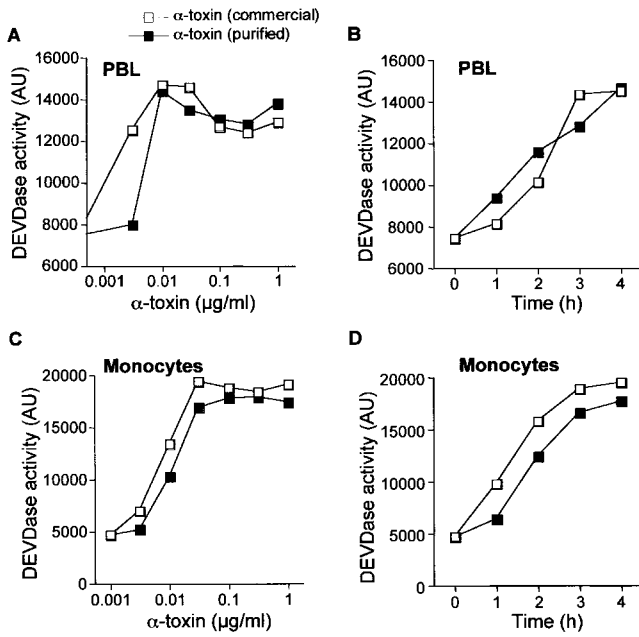
Figure 5. *S. aureus* α-toxin induces caspase-3 activation in human PBL and monocytes. DEVDase activity in extracts of PBL (A and B) and monocytes (C and D). Cells were either incubated for 4 h with the indicated α-toxin concentrations (A and C) or with 30 ng/ml α-toxin for the indicated times (B and D).
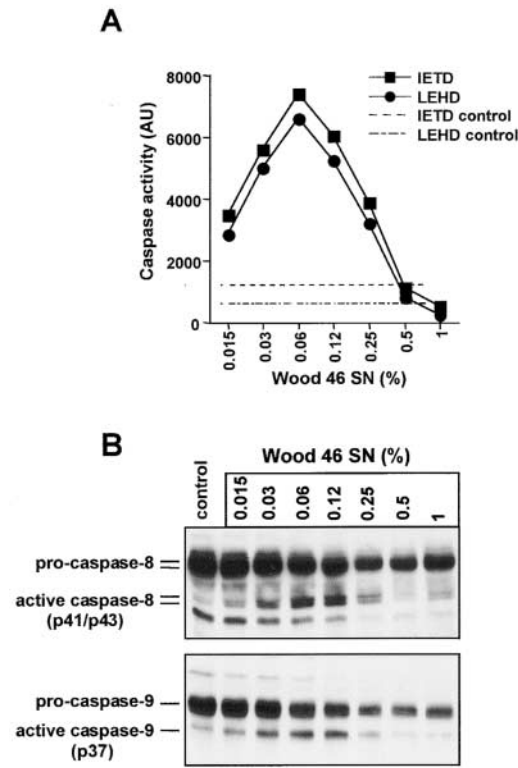

Figure 6. *S. aureus* induces activation of the initiator caspases 8 and 9. (A) Jurkat cells were incubated with the indicated dilutions of Wood 46 supernatant (SN). After 4 h, cell extracts were prepared and analyzed for caspase activity using the fluorogenic substrates IETD AMC for caspase 8 (■) and LEHD AMC for caspase 9 (●). The two dashed lines represent IETDase and LEHDase activity in untreated cells. (B) Western blot analysis of the status of caspase-8 (top) and caspase-9 (bottom) in Jurkat cells that were either left untreated (control) or incubated for 4 h with the indicated dilutions of Wood 46 supernatant (SN). The uncleaved proforms and the p43/p41 fragments of caspase-8 and the p37 fragment of caspase-9 are indicated.

dose-dependent increase of IETDase and LEHDase activity in Jurkat cells (Fig. 6 A). These enzymatic activities correlated well with the appearance of the p41/p43-processed caspase-8 subunits (Fig. 6 B, top) and the p37 active caspase-9 fragment, respectively (Fig. 6 B, bottom). Wood 46–induced caspase-8 and -9 activation reached a peak when 0.06% of the supernatant was used and declined rapidly with higher concentrations, which is reminiscent of the activation profile of caspase-3 (Fig. 3 C and Fig. 4 A). Similar results were obtained with purified α-toxin (unpublished data), indicating that both caspase-8 and caspase-9 are involved in *S. aureus–*mediated cytotoxicity.

S. aureus mediates cell death and caspase-8 activation independently of CD95 and death receptor signaling

Caspase-8 has been identified originally as the most apical caspase in apoptosis mediated by the surface receptor CD95. Therefore, we asked whether a functional CD95 pathway is essential for *S. aureus–*induced caspase-8 activation and T cell apoptosis. For this purpose, Jurkat subclones with various deficiencies in crucial CD95 signaling components that rendered these cells resistant towards CD95-mediated apoptosis (Fig. 7 B) were analyzed for their capability to undergo cell death after treatment with supernatant of the *S. aureus* strain RN6390. Like Jurkat wild-type cells, Jurkat-R cells that have lost CD95 expression due to continuous culture with the agonistic anti-CD95 antibody were killed efficiently by RN6390 in a dose-dependent manner (Fig. 7 A). Also FADD- or caspase-8–deficient Jurkat cells were killed to a similar extent compared with Jurkat wild-type cells (Fig. 7 A). Together, these findings demonstrate that neither CD95/CD95L interaction nor other FADD-dependent

death receptors are required for *S. aureus–*induced apoptosis and even indicate that caspase-8 itself, although activated, may not be essential for this process.

Next, we examined the influence of the CD95 signaling pathway on the *S. aureus–*mediated activation of the effector caspase-3. As shown in Fig. 7 C, activation of caspase-3 was also achieved via a CD95-independent pathway, since RN6390 induced DEVDase activity in Jurkat-R cells and even in caspase-8–deficient cells to a similar extent as in Jurkat wild-type cells. Only FADD-deficient cells displayed a slight delay in RN6390-induced caspase-3 activation (Fig. 7 C). Similar results were obtained with supernatant of the *S. aureus* strain Wood 46 and purified α-toxin (unpublished data). As a control, the various Jurkat subclones were treated with the anti-CD95 antibody, which did not result in an enhancement of DEVDase activity, confirming that these components are essential for caspase-3 activation in the CD95 signaling pathway (Fig. 7 D). In contrast, the chemotherapeutic drug etoposide, which is known to mediate apoptosis independently of the death receptor pathway, induced similar levels of caspase-3 activity in all Jurkat sublines, ruling out a general defect in the apoptosis pathways in these cells (Fig. 7 D).
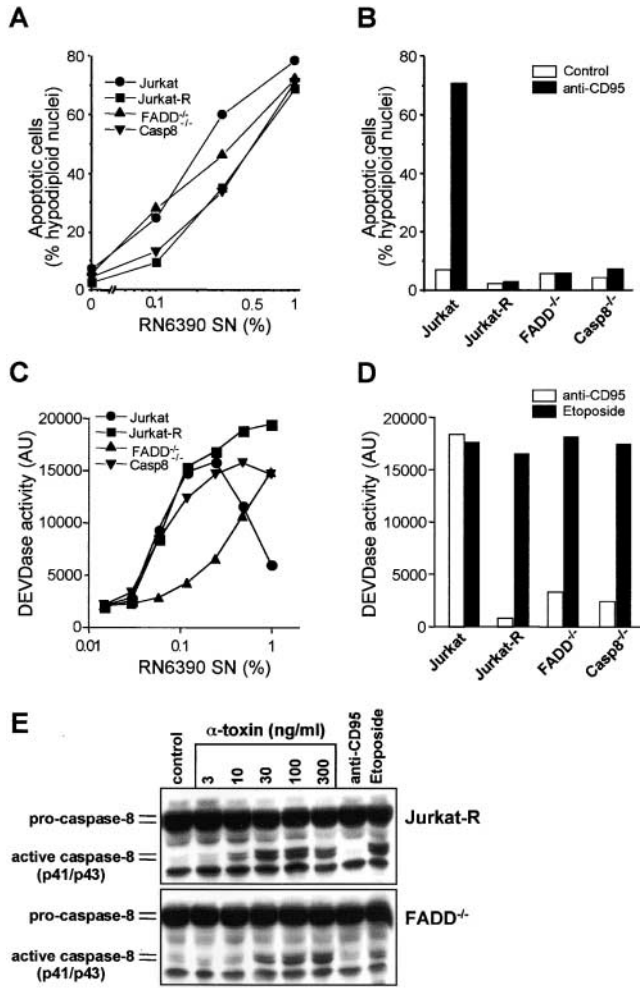
Figure 7. **S. aureus α-toxin signals cell death and caspase activation independently of death receptor pathways.** Assessment of cell death (A and B) and DEVDase activity (C and D) in Jurkat cells (●), Jurkat-R cells (■), FADD-deficient Jurkat cells (▲), and caspase-8–deficient Jurkat cells (▼). Cells were incubated with increasing concentrations of RN6390 supernatant (A and C), anti-CD95 (B and D), or etoposide (D). (E) Western blot analyses demonstrating the activation of caspase-8 in Jurkat-R cells (top) and FADD-deficient cells (bottom) that were either left untreated (control) or incubated for 4 h with the indicated concentrations of α-toxin. As a control, cells were treated for 4 h with anti-CD95 or etoposide. The blots show the uncleaved precursor forms and the p43/p41 intermediate fragments of caspase-8.

Although we have shown that the CD95 signaling pathway is not required for *S. aureus*–induced cell death, caspase-8 is activated and hence appears to be involved (Fig. 6). To solve this apparent discrepancy, we investigated more closely the role of caspase-8 in this process. As demonstrated by Western blot analyses, α-toxin (Fig. 7 E) and supernatant of the *S. aureus* strain Wood 46 (unpublished data) induced caspase-8 processing into the p41/p43 subunits in both Jurkat-R and FADD-deficient cells in a similar dose-dependent manner as observed in Jurkat wild-type cells (Fig. 6 B). These results clearly indicate that a functional CD95 death pathway is not required for *S. aureus*– and α-toxin–mediated caspase-8 activation. As expected, etoposide also induced caspase-8 processing in both Jurkat-R– and Jurkat

FADD-deficient cells, whereas treatment of the cells with the agonistic anti-CD95 antibody had no effect (Fig. 7 E).

Bcl-2 inhibits *S. aureus*–mediated cell death and caspase activation

Next, we examined the possibility that caspase activation by *S. aureus* is mediated via the intrinsic death pathway. For this purpose, Jurkat cells stably expressing the antiapoptotic protein Bcl-2 that is known to prevent mitochondrial cytochrome c release were incubated with increasing concentrations of Wood 46 (Fig. 8 A) or RN6390 (Fig. 8 B) supernatants. Cell death was inhibited dose dependently in Jurkat Bcl-2 cells compared with similarly treated Jurkat vector cells. However, cell death inhibition was observed only when low concentrations of *S. aureus* culture supernatants were used, whereas cell death induced with higher concentrations of Wood 46 or RN6390 supernatants was not affected by Bcl-2. This is consistent with our previous results, demonstrating that only low concentra-
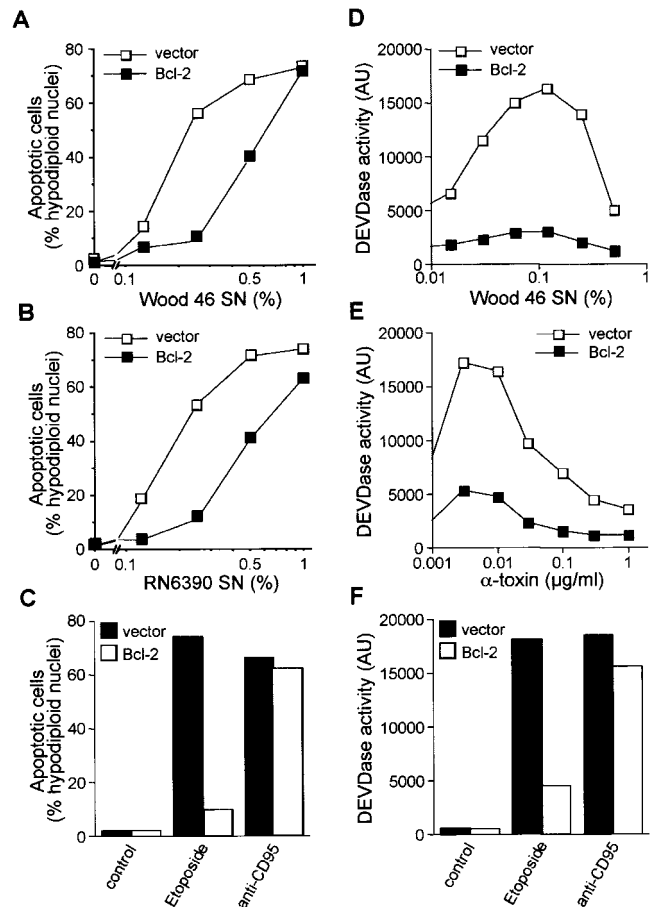

Figure 8. **S. aureus mediates cell death and caspase activation via the Bcl-2–controlled mitochondrial death pathway.** Assessment of cell death (A and B) and caspase-3–like activity (D and E) in Jurkat vector control cells (□) or cells overexpressing Bcl-2 (■). Cells were incubated with increasing concentrations of Wood 46 supernatant (A and D), RN6390 supernatant (B), or α-toxin (E). Assessment of etoposide- or CD95-induced apoptosis (C) and DEVDase activity (F) in vector cells (filled bars) and Bcl-2 cells (open bars) served as controls. Cell death and DEVDase activity were determined after 24 and 4 h, respectively.

tions of *S. aureus* culture supernatants induce caspase activation (Figs. 3, 4, and 6).

Similarly, almost no DEVDase activity could be detected in Bcl-2–overexpressing Jurkat cells treated with supernatant of Wood 46 (Fig. 8 D) or α-toxin (Fig. 8 E), confirming that *S. aureus*–induced caspase activation and cell death are mediated via the mitochondrial pathway. As a control, etoposide-induced apoptosis and caspase activation that require the intrinsic death pathway were almost completely abrogated in Bcl-2–overexpressing cells, whereas CD95-mediated signaling was not affected (Fig. 8, C and F).

α-Toxin induces cytochrome c release in intact cells and isolated mitochondria

To further examine the *S. aureus*–induced caspase activation pathway, we monitored the release of mitochondrial cytochrome c from Jurkat cells incubated for various times with α-toxin. Western blot analysis showed a time-dependent increase of cytochrome c release, which was first, although only marginally, visible after a 2-h exposure of the cells to α-toxin (Fig. 9 A). Nevertheless, continuous exposure of the cells to α-toxin resulted in increasing cytosolic cytochrome c levels that were comparable to those released after an 8-h treatment of cells with the kinase inhibitor staurosporine.

We then asked whether α-toxin has a direct effect on mitochondria. Isolated mitochondria from Jurkat cells were incubated for 30 min with various concentrations of α-toxin, and the supernatants were analyzed by Western blotting for their cytochrome c content. As shown in Fig. 9 B, α-toxin induced a dose-dependent release of cytochrome c from isolated mitochondria. The amount of cytochrome c released after α-toxin treatment was comparable to the level of cytochrome c release achieved by incubation of the mitochondria with betulinic acid, a potent cell death inducer with a direct effect on mitochondria (Fulda et al., 1998). No cytochrome c was released when the mitochondria were treated with supernatant of the non–cytotoxic *S. carnosus* strain TM300 (unpublished data). More importantly, both betulinic acid and α-toxin did not induce cytochrome c release from mitochondria isolated from Bcl-2–overexpressing Jurkat cells (Fig. 9 B), demonstrating that α-toxin can directly target mitochondria in a Bcl-2–controlled manner. Interestingly, Bcl-2 prevented breakdown of the mitochondrial membrane potential only when low doses of α-toxin were used but had a weaker effect with high α-toxin concentrations (Fig. 9 C). Nevertheless, our data clearly demonstrate that α-toxin is a mediator of *S. aureus*–induced cell death that triggers caspase activation in Jurkat T cells via mitochondrial cytochrome c release independently of death receptor pathways.

Discussion

Several pathogens, including *Salmonella typhimurium*, *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Listeria monocytogenes*, and *Yersinia enterocolitica*, are able to trigger apoptosis of mammalian host cells, whereas others such as *Rickettsia spp.* and *Chlamydia spp.* have been reported to exert antiapoptotic effects (Weinrauch and Zychlinsky, 1999; Gao and Kwaik, 2000). However, in most cases the underlying molecular processes and their relevance to host pathogen inter-
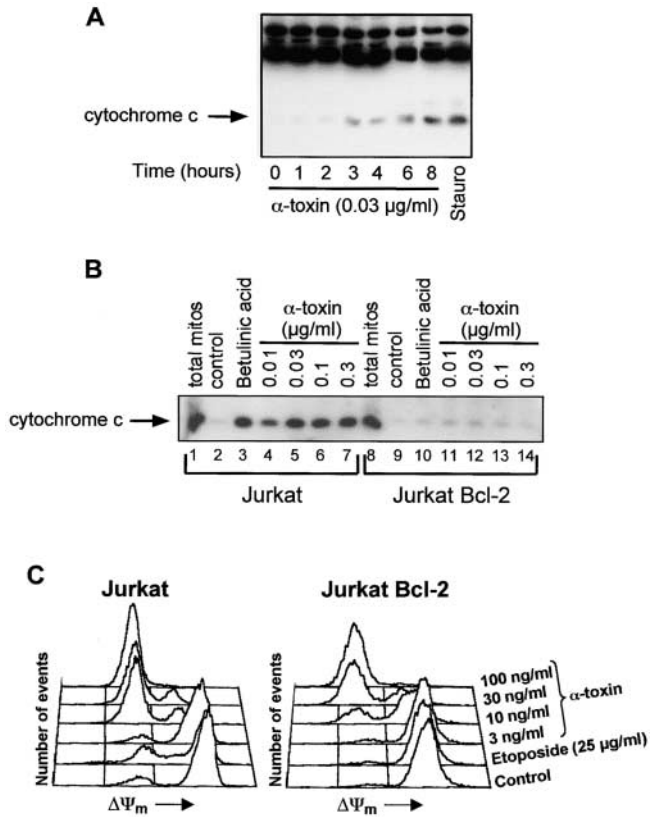

Figure 9.   *S. aureus* α-toxin induces cytochrome c release in intact cells and isolated mitochondria in a Bcl-2–controlled manner. (A) Western blot analysis of cytosolic cytochrome c in Jurkat cells treated for the indicated times with 0.03 μg/ml α-toxin or for 8 h with 1 μg/ml staurosporine (Stauro). (B) Effect of α-toxin on isolated mitochondria. Isolated mitochondria from Jurkat (lanes 1–7) or from Bcl-2–overexpressing Jurkat cells (lanes 8–14) were incubated for 30 min with a buffer control (lanes 2 and 9), 10 μg/ml betulinic acid (lanes 3 and 10), or the indicated concentrations of α-toxin (lanes 4–7 and 11–14). The release of cytochrome c was detected by immunoblotting. Lanes 1 and 8 (total mitos) represent the complete amount of cytochrome c present in mitochondria from Jurkat and Jurkat Bcl-2 cells, respectively. (C) Measurement of the mitochondrial transmembrane potential ($\Delta\Psi_m$) in untreated (Control) Jurkat and Jurkat Bcl-2 cells or in cells treated for 4 h with etoposide or the indicated concentrations of α-toxin.

actions remain largely unknown. In the present study, we have delineated the mechanisms of *S. aureus*–induced T cell apoptosis. We show that apoptosis in response to *S. aureus* infection can be triggered by a soluble factor, α-toxin, which mediates caspase activation via the mitochondrial apoptosis pathway. Interestingly, α-toxin was able to induce cytochrome c release not only in intact cells but also from isolated mitochondria in a Bcl-2–controlled manner, suggesting that at least part of its cytotoxicity is mediated by a direct proapoptotic mechanism.

The ability of *S. aureus* to induce apoptosis has been noticed previously in a variety of cell types including epithelial cells, endothelial cells, keratinocytes, osteoblasts, lymphocytes, and macrophages (Jonas et al., 1994; Bayles et al., 1998; Menzies and Kourteva, 1998, 2000; Kahl et al., 2000, Nuzzo et al., 2000; Tucker et al., 2000). Induction of apoptosis by *S. aureus* may cause tissue damage, compromise the

antimicrobial immune response, and thereby promote bacterial spread. Although *S. aureus* is classically considered as an extracellular pathogen, it can invade a number of different cell types (Sinha et al., 1999). In some cell types, such as epithelial cells, keratinocytes, or osteoblasts, *S. aureus*–induced apoptosis has been proposed to require the prior internalization of bacteria (Bayles et al., 1998; Menzies and Kourteva, 1998; Kahl et al., 2000, Nuzzo et al., 2000; Tucker et al., 2000). Contrary to these reports, our data clearly show that in T cells bacterial invasion is not required and that soluble α-toxin is a mediator of *S. aureus*–induced apoptosis. This finding is supported by several independent observations: (a) we noticed that the cytotoxicity of different *S. aureus* strains correlated with the expression levels of α-toxin; (b) we show that an identical pattern of apoptosis was obtained using either intact bacteria, bacterial supernatants, or α-toxin; (c) apoptosis induction did not occur with the bacterial supernatant of an α-toxin knock-out strain; and most importantly (d) the cytotoxic activity of α-toxin or the bacterial supernatants was abolished completely by two different neutralizing α-toxin antibodies. Although we detected minor impurities in the commercially available α-toxin, this preparation induced cell death and caspase activation with an almost identical dose dependency as achieved with highly purified α-toxin. Moreover, both α-toxin preparations efficiently induced caspase-3 activation in human PBLs and monocytes with a similar dose dependency. Thus, our data are in line with a recent report suggesting that α-toxin is necessary for apoptosis induction also in endothelial cells (Menzies and Kourteva, 2000) and thereby underscore the pathophysiological function of *S. aureus* α-toxin, which might explain the death of immune cells during sepsis. However, since the cellular systems used in this study might not accurately reflect the host–parasite interactions in an infected animal we cannot exclude additional staphylococcal exoproteins that might contribute to cell death induction in vivo.

Interestingly, we found that caspase activation and subsequent DNA fragmentation were only triggered at low concentrations of α-toxin, whereas higher concentrations did not induce apoptotic alterations. These differences may be directly related to the molecular properties of α-toxin. At low doses, the toxin binds to specific as yet unidentified cell surface receptors and produces small heptameric pores that selectively facilitate the release of monovalent ions, resulting in DNA fragmentation and apoptosis (Jonas et al., 1994; Song et al., 1996; Valeva et al., 1997). However, at high doses (>6 μg/ml) α-toxin nonspecifically adsorbs to the lipid bilayer, forming larger pores that are Ca^{2+} permissive, which results in massive necrosis. Moreover, the larger pores will also lead to the loss of ATP, which is required for most apoptotic processes (Leist et al., 1997; Ferrari et al., 1998). Thus, the modes of cell death may critically depend on the concentration of α-toxin.

S. aureus and α-toxin did not only induce the activation of effector caspase-3 but also of caspase-8 and -9, the two major initiator caspases. The activation of caspase-8 might be indicative for the involvement of CD95 as suggested recently for *S. aureus*–induced apoptosis in mammary epithelial cells (Wesson et al., 2000). Moreover, *S. aureus*–induced apoptosis of monocytes has been proposed to involve CD95

signaling caused by the upregulation of CD95 ligand expression (Baran et al., 2001). However, in three different Jurkat cell clones that are completely resistant against CD95 but sensitive towards anticancer drug-induced apoptosis (this study; Wesselborg et al., 1999; Engels et al., 2000), α-toxin induced apoptosis with a similar dose response as in parental cells. Thus, in contrast to other bacteria, including *Pseudomonas aeruginosa* (Grassme et al., 2000) and *Helicobacter pylori* (Rudi et al., 1998), our data clearly indicate that neither CD95 or other FADD-dependent death receptors are required for *S. aureus*–induced cytotoxicity in T cells.

Since death receptors were obviously not involved in *S. aureus*–induced cell death, we investigated the role of mitochondria. An early event in this pathway is the proapoptotic translocation of mitochondrial cytochrome c into the cytosol, which is inhibited by antiapoptotic Bcl-2 proteins (Kluck et al., 1997; Yang et al., 1997). Indeed, in cells overexpressing Bcl-2, *S. aureus*–mediated apoptosis and caspase activation were suppressed significantly compared with control cells. Moreover, *S. aureus* α-toxin caused a time-dependent redistribution of cytochrome c, which was associated with proteolytic processing of caspase-9 and subsequent activation of effector caspases. The mitochondrial activation of caspase-9 and -3 was also presumably responsible for the activation of caspase-8 by *S. aureus*. We have shown recently that during drug-induced apoptosis caspase-8 activation is a postmitochondrial event downstream of caspase-3 (Engels et al., 2000). In this scenario, caspase-8 might induce Bid activation and enforce cytochrome c release, thereby amplifying the apoptotic cascade.

A surprising observation was the fact that α-toxin did not only induce cytochrome c release in intact cells but also from isolated mitochondria in a Bcl-2–controlled manner. A rapid and dose-dependent release of cytochrome c from isolated mitochondria was observed already with low doses of α-toxin, whereas supernatants of the bacterial strain TM300 that failed to induce apoptosis also did not induce cytochrome c release in vitro. Interestingly, caspase activation, DNA fragmentation, and cytochrome c release induced with low α-toxin doses were blocked completely by Bcl-2, whereas cell death induced with high α-toxin concentrations proceeded independently of mitochondria-mediated caspase activation. The reason for this phenomenon is presently unknown but is consistent with our observation that also breakdown of the mitochondrial membrane potential was prevented only by Bcl-2 when low α-toxin doses were employed. This latter finding is in agreement with several reports showing that the loss of the mitochondrial membrane potential is a late event in apoptosis not associated necessarily with cytochrome c release (Bossy-Wetzel et al., 1998; Goldstein et al., 2000; Kroemer and Reed, 2000). Nonetheless, our data suggest that α-toxin may directly target mitochondria, a mechanism that has also been described recently for VacA, the vacuolating cytotoxin of *Helicobacter pylori* (Galmiche et al., 2000) and the *Neisseria gonorrhoeae* porin PorB (Müller et al., 2000). It is interesting to note that *N. gonorrhoeae* PorB and *S. aureus* α-toxin have similar three-dimensional structures consisting of antiparallel β-barrels; both toxins also have the ability to insert necessarily into artificial planar lipid membranes (Song et al., 1996; Boya et al., 2001).

Therefore, our results suggest that *S. aureus* α-toxin might induce apoptosis by two separate mechanisms. First, insertion of α-toxin into the plasma membrane and subsequent pore formation will lead to K^+ efflux and Na^+ influx. The depletion of potassium ions may cause caspase activation as it has been observed in monocytes treated with depolarizing drugs such as nigericin (Warny and Kelly, 1999). Secondly, α-toxin might translocate to the outer mitochondrial membrane and trigger cytochrome c release. One may speculate that the direct targeting of mitochondria by α-toxin may be important, particularly in those cell types in which apoptosis requires the prior internalization of *S. aureus*.

In conclusion, our data demonstrate that *S. aureus* α-toxin induces caspase activation and apoptosis through mitochondrial cytochrome c release independently of death receptor signaling. These findings open promising insights into how *S. aureus* mediates cytotoxicity and may provide a molecular rationale for future therapeutic interventions in bacterial infections.

Materials and methods

Jurkat cell lines, reagents, and antibodies
All cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM glutamine, and 50 μg/ml of each streptomycin and penicillin/ml (all from GIBCO BRL). The Jurkat sublines Jurkat-R and the FADD- and caspase-8–deficient Jurkat cells were described previously (Juo et al., 1998; Wesselborg et al., 1999). Jurkat cells stably transfected with Bcl-2 or empty vector were from H. Walczak (Deutsche Krebsforschungszentrum, Heidelberg, Germany). Aprotinin, antipain, pepstatin, leupeptin, and PMSF, etoposide, RNase A, proteinase K, *S. aureus* α-toxin, and the neutralizing polyclonal rabbit anti–α-toxin antibody were purchased from Sigma-Aldrich. The anti-CD95 mAb (IgG3; BioCheck) and etoposide were used at concentrations of 1 μg/ml and 100 μM, respectively. The highly purified α-toxin preparation was a generous gift from A. Valeva and S. Bhakdi (Institute of Medical Microbiology and Hygiene, Mainz, Germany). Recombinant lysostaphin was obtained from WAK Chemie/Applied Microbiology. Human serum albumin was from Behring. The fluorogenic caspase substrates DEVD-AMC, IETD-AMC, and LEHD-AMC were from Biomol. Neutralizing polyclonal sheep anti–α-toxin was from Toxin Technology, and monoclonal anti–caspase-8 was from BioCheck. The polyclonal goat and rabbit antibodies recognizing caspase-3 and caspase-9 were from R&D Systems and New England Biolabs, Inc., respectively. Anti–α-fodrin (mAb1622) was purchased from Chemicon, and anti–cytochrome c (mAb7H8.2C12) was from PharMingen.

Isolation and culture of PBL and monocytes
Human PBLs and monocytes were isolated by standard Ficoll-Paque (Amersham Pharmacia Biotech) and subsequent Percoll gradient centrifugation from heparin-treated blood of healthy donors. Purity of monocytes and lymphocytes was confirmed by FACS® analyses using CD14 and CD3 antibodies, respectively, and was >90%.

Bacterial strains, cultures, and supernatants
The properties and source of all the bacterial strains used in this study are listed in Table I. DU5883 is an isogenic mutant of 8325-4 with the dele-

tions (by allelic replacement) in fnbA and fnbB encoding fibronectin-binding proteins A and B, respectively. DU1090 is an isogenic mutant of 8325-4 with a deletion (by allelic replacement) in hla, the gene for α-toxin. All mutants were provided by T. Foster (Moyne Institute of Preventive Medicine, Dublin, Ireland). Bacteria were grown in 5 ml of brain-heart-infusion broth (Merck) in 25-ml glass tubes on a rotatory shaker (200 rpm) at 37°C for 12–14 h and pelleted for 5 min at 3,350 *g*. Supernatants were filtered through a Millex-GP filter unit (0.22 μm; Millipore), resuspended in PBS/1% human serum albumin, and kept on ice. Sterility and actual concentration of viable bacteria were assayed by plating appropriate serial dilutions on tryptic soy agar plates followed by manual counting.

Preparation of cell extracts and Western blotting
Cell extracts were prepared as described (Jänicke et al., 1998a). To confirm equal loading, protein concentrations were determined with the Bio-Rad Laboratories protein assay. Proteins were separated in SDS-polyacrylamide gels and subjected to Western blotting. The proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Pharmacia Biotech).

Detection of DNA fragmentation
The proportion of nuclei containing hypodiploid DNA was assessed after 24 h by the method of Nicoletti et al. (1991) with minor modifications as described (Wesselborg et al., 1999; Engels et al., 2000).

DNA fragmentation was also assessed using agarose gel electrophoresis. DNA was prepared by lysing 5×10^6 Jurkat cells in 0.2 ml of $0.5 \times$ TE buffer (20 mM Tris, 0.5 mM EDTA, pH 8.0) containing 0.25% NP-40 and 50 μg RNase A at 37°C for 30 min. Proteinase K (200 μg/sample) was added, and the samples were incubated for another 30 min at 37°C. DNA sample buffer was added, and aliquots were analyzed on 1.6% agarose gels.

Fluorometric determination of caspase-3, -8, or -9 activities
Caspase activities were determined by incubation of cell lysates with 50 μM of the fluorogenic substrates DEVD-AMC for caspase-3, IETD-AMC for caspase-8, or LEHD-AMC for caspase-9 in 200 μl buffer containing 50 mM Hepes, pH 7.4, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, and 10 mM DTT. The release of aminomethyl-coumarin was measured by fluorometry (FL600; Bio-Tek Instruments) using an excitation wavelength of 360 nm and an emission wavelength of 475 nm. The catalytic activities are given in arbitrary units (AU).

Isolation of the mitochondria-enriched heavy membrane fraction and measurement of cytochrome c release
Enrichment of mitochondria was achieved by permeabilizing 2×10^8 Jurkat cells for 5 min in a buffer containing 100 μg/ml digitonin, 250 mM sucrose, 20 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml of each of the protease inhibitors aprotinin, pepstatin, and leupeptin. Cells were centrifuged at 200 *g* for 10 min at 4°C to remove cell nuclei. The supernatant was transferred to a fresh tube and centrifuged at 10,000 *g* for 15 min at 4°C. The pelleted mitochondria were washed twice with the same buffer without digitonin, EGTA, and EDTA. 10 μl of the mitochondria suspension were incubated for 30 min at 37°C with the indicated stimuli and pelleted by centrifugation. The supernatants were loaded on a 15% SDS-polyacrylamide gel and analyzed by immunoblotting. To monitor cytochrome c release in Jurkat cells, ∼4×10^6 cells were cultured for the indicated times in the presence or absence of 0.03 μg/ml α-toxin, washed, and resuspended in 200 μl of the above mentioned buffer. After 5 min at 4°C, cells were centrifuged at 1,000 *g* for 10 min to remove cell nuclei

Table I. **Bacterial strains used in this study**

Strain	Relevant genotype	Properties	Source
S. aureus			
Cowan I		National Collection of Type Cultures 8530 (septic arthritis)	American Type Culture Collection 12589
8325-4		National Collection of Type Cultures 8325 cured of prophages	McDevitt et al., 1994; Greene et al., 1995
DU5883	8325-4 *fnbA fnbB*	FnBPA -, FnBPB -	Greene et al., 1995
DU1090	8325-4 *hla*	α-Toxin -	O'Reilly et al., 1986
RN6390		Wild-type 8325-4 strain	Novick et al., 1993
Wood 46		Strong α-toxin producer, protein A -	National Collection of Type Cultures 10344
6850		Clinical isolate (osteomyelitis)	Balwit et al., 1994
S. carnosus			
TM300		No expression of toxins	Schleifer and Fischer, 1982

and at 10,000 *g* for 15 min to remove mitochondria. The resulting supernatants were analyzed for cytochrome c content as described above.

Determination of the mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analyzed using the $\Delta\Psi_m$-specific stain TMRE (Molecular Probes). In brief, 10^5 cells were stained after 4 h in a solution containing 25 nM TMRE for 10 min. Staining was quantified by FL2 and scatter characteristics using a flow cytometer.

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