

The mitochondrial protein Bak is pivotal for gliotoxin-induced apoptosis and a critical host factor of *Aspergillus fumigatus* virulence in mice

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A *Aspergillus fumigatus* infections cause high levels of morbidity and mortality in immunocompromised patients. Gliotoxin (GT), a secondary metabolite, is cytotoxic for mammalian cells, but the molecular basis and biological relevance of this toxicity remain speculative. We show that GT induces apoptotic cell death by activating the proapoptotic Bcl-2 family member Bak, but not Bax, to elicit the generation of reactive oxygen species, the mitochondrial release of apoptogenic factors, and caspase-3 activation. Activation of Bak by GT is

direct, as GT triggers in vitro a dose-dependent release of cytochrome c from purified mitochondria isolated from wild-type and Bax- but not Bak-deficient cells. Resistance to *A. fumigatus* of mice lacking Bak compared to wild-type mice demonstrates the in vivo relevance of this GT-induced apoptotic pathway involving Bak and suggests a correlation between GT production and virulence. The elucidation of the molecular basis opens new strategies for the development of therapeutic regimens to combat *A. fumigatus* and related fungal infections.

Introduction

The saprophytic fungus *Aspergillus fumigatus* is a serious health hazard in hospitals (Latge, 1999, 2001). *A. fumigatus* is responsible for >90% of invasive aspergilloses (IA), with mostly fatal outcomes in immunocompromised patients suffering from AIDS, tuberculosis, cancer, or bone marrow/organ transplants (Latge, 1999; Bauters et al., 2005). The true incidence of *A. fumigatus* infections is underestimated because of the inherent difficulty of positive diagnosis (Latge, 1999).

Second-generation anti-fungals, such as amphotericin B lipid forms, echinocandins, and azoles, have only shown modest improvements in efficacy and at lower toxicity. Thus, anti-

A. fumigatus therapy remains inadequate and, as a consequence, high morbidity and mortality from IA prevails (Latge, 1999; Bauters et al., 2005). One reason for this is our poor understanding of the pathobiology of *A. fumigatus*. A multitude of putative *A. fumigatus* virulence factors, such as extracellular metalloprotease, serine protease, aspartic protease, catalase, phospholipases, haemolysin, and the cytotoxin ASPF1 have been implicated in IA, but none has yet been shown to be involved in the pathogenesis of *A. fumigatus* in experimentally induced infections (Latge, 2001).

Gliotoxin (GT), an abundant mycotoxin produced by *A. fumigatus* and other fungi, such as *Candida albicans*, belongs to the epipolythiodioxopiperazine class of secondary metabolites (Taylor, 1971) and is characterized by a reactive disulfide bridge across the piperazine ring (Mullbacher et al., 1986). GT has been proposed to constitute a virulence factor in IA because of its immunosuppressive properties (Eichner and Mullbacher, 1984; Mullbacher and Eichner, 1984). GT has been shown in vitro to inhibit multiple processes associated

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Abbreviations used in this paper: 2-HE, 2-hydroxiethidine; AIF, apoptosis-inducing factor; ANT, adenine nucleotide transporter; FDM, factor-dependent myeloid; GT, gliotoxin; IA, invasive aspergilloses; MEF, mouse embryonic fibroblast; MnTBAP, Mn(III) tetrakis(4-benzoic acid) porphyrin chloride; MPTP, mitochondrial permeability transition pore; NAC, N-acetylcysteine; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; wt, wild-type.

with activation, differentiation, and/or effector functions of immune cells (Mullbacher et al., 1987; Waring et al., 1988a; Gardiner et al., 2005). This includes activation of NF- κ B (Pahl et al., 1996), neutrophil and macrophage oxidative killing (Mullbacher et al., 1985; Murayama et al., 1996; Tsunawaki et al., 2004), polymorphonuclear neutrophils chemotaxis (Shah et al., 1998), polymorphonuclear neutrophils and macrophage phagocytosis (Mullbacher et al., 1985; Eichner et al., 1986; Murayama et al., 1996; Shah et al., 1998), activation of cytolytic T cells (Mullbacher and Eichner, 1984; Waring et al., 1988a), and IFN γ production by CD4⁺ lymphocytes (Wichmann et al., 2002). Most important, GT was found to induce mammalian cell apoptosis (Waring et al., 1988b; Sutton et al., 1994) accompanied by the production of reactive oxygen species (ROS) and mitochondrial membrane disruption (Eichner et al., 1988; Zhou et al., 2000; Suen et al., 2001; Kweon et al., 2003). The proposition that GT is a virulence factor is supported by more recent findings demonstrating that GT is expressed *in vivo* during experimental and human aspergillosis (Lewis et al., 2005) and that the decreased levels of pulmonary GT observed with an *A. fumigatus* mutant defective in *LaeA*, a global regulator of secondary metabolism, is associated with impaired virulence (Bok et al., 2005; unpublished data). However, it should be noted that *LaeA* regulates expression of a cassette of genes, including GT and other secondary metabolites. Thus, the virulence of the wild-type (wt) strain of *A. fumigatus* is not necessarily linked to GT alone.

Definitive evidence on the molecular basis of GT-mediated apoptosis and its relevance in the parasitized vertebrate host is still lacking. Many diverse stimuli, including irradiation, toxic drugs, and pathogens, transduce apoptotic signals to mammalian cells, resulting in the disruption of mitochondrial membranes (Kroemer and Reed, 2000; Dockrell, 2001; Green and Kroemer, 2004) and the subsequent release of apoptotic factors, such as cytochrome *c* and apoptosis-inducing factor (AIF; Liu et al., 1996; Susin et al., 1999; Pardo et al., 2001). Complex formation of cytochrome *c* with Apaf-1 and caspase-9 leads to further induction of effector caspases, i.e., caspase-3 and -7 (Li et al., 1997). Permeabilizing the mitochondrial membranes by diverse stimuli strictly

depends on the proapoptotic Bcl-2 family members Bak and Bax (Marzo et al., 1998; Shimizu et al., 1999). This is indicated by the fact that deficiency in Bax and Bak renders cells resistant to numerous apoptotic stimuli (Wei et al., 2000, 2001). Although how Bax and Bak are regulated is still debated, it has become clear that they are somehow activated by BH3-only proteins, which trigger their conformational change, oligomerization, and pore forming activity (Letai et al., 2002; Willis et al., 2005). Among BH3-only molecules, Bid and Bim may directly activate Bax and Bak by a hit-and-run mechanism (Kuwana et al., 2005), whereas other members of this family seem to act by antagonizing the survival activity of antiapoptotic proteins, i.e., Bcl-2, Bcl-x_L, and Mcl-1 (Huang and Strasser, 2000; Chen et al., 2005; Willis et al., 2005). However, it has not yet been studied by which mediators/ effectors GT induces mitochondrial membrane disruption and apoptosis.

We show now for the first time that Bak, which constitutively resides on mitochondria (Griffiths et al., 1999), is the primary intracellular target in GT-mediated and ROS-facilitated apoptosis *in vitro* and that in a corticosteroid-based IA model, a knockout mouse strain lacking Bak is more resistant to *A. fumigatus* than wt mice.

Results

GT induces apoptosis in mouse embryonic fibroblast (MEF) cells

As GT may induce apoptosis and necrosis in mammalian cells, depending on the concentration of GT and the target cell used (Braithwaite et al., 1987; Hurme et al., 2002; Kweon et al., 2003; Orr et al., 2004), we first established optimal conditions that allowed us to readily monitor proapoptotic processes in MEFs. As shown in Fig. 1 A, 1 μ M GT induced nuclear fragmentation in the majority of wt MEFs. Furthermore, ~50% of these cells were apoptotic, i.e., had phosphatidylserine (PS) exposed (annexin V staining) without plasma membrane disruption, whereas the rest of the cells already showed secondary necrosis (loss of membrane integrity as shown by propidium iodide [PI] staining; Fig. 1 B; Pardo et al., 2004). Thus, 1 μ M GT was used in all subsequent experiments.

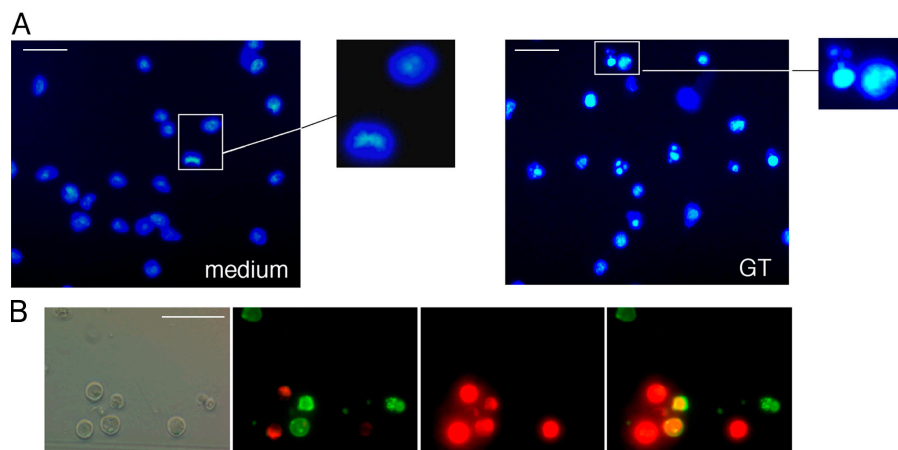


Figure 1. **GT induces apoptosis in MEFs.** wt MEFs were incubated with or without 1 μ M GT for 4 h and stained with Hoechst 33342 (A) or annexin V-FITC plus PI (B), and fluorescence images were taken as described in Materials and methods. Bars: (A) 25 μ m; (B) 40 μ m.

GT-induced apoptosis depends on Bak but not Bax and/or Bid

We next compared GT-induced PS exposure and PI staining between wt and knockout ($-/-$) MEFs. In addition, we studied the impact of GT on the $\Delta\psi_m$ and the production of ROS in these cells. As shown in Fig. 2 A, GT significantly increased the number of annexin V/PI-positive cells in wt, $Bax^{-/-}$, and $Bid^{-/-}$ MEFs as compared with mock-treated cells, whereas $Bak^{-/-}$ and $Bak^{-/-} \times Bax^{-/-}$ MEFs did not. Similarly, GT treatment led to a significant reduction in the mitochondrial membrane potential ($\Delta\psi_m$) and a parallel increase in ROS production in wt, $Bax^{-/-}$, and $Bid^{-/-}$, but not in $Bak^{-/-}$ and $Bak^{-/-} \times Bax^{-/-}$ MEFs (Fig. 2 B). It is our experience that there is considerable variation in the numbers of cells induced to express the respective proapoptotic markers from experiment to experiment. However, the differentials between experimental and control groups in individual experiments were always highly significant. This suggests that in MEFs, Bak, but not Bax, is critical for GT-induced loss of plasma membrane integrity and the $\Delta\psi_m$. Moreover, Bid, which is known to activate Bak and Bax during apoptosis (Wei et al., 2001; Letai et al., 2002; Kuwana et al., 2005), seems to be dispensable for these processes. In support of a key role of Bak in GT-mediated cell death, we found that trypan blue exclusion was significantly reduced only in $Bak^{-/-}$ and $Bak^{-/-} \times Bax^{-/-}$, but not in wt or $Bax^{-/-}$ MEFs (Fig. 2 D). In contrast to GT-induced apoptosis, the apoptosis-inducing drug staurosporine induced reduction in the $\Delta\psi_m$ and a parallel increase in ROS, which was only prevented in the absence of both Bak and Bax, as already described (Fig. 2 C; Wei et al., 2001).

GT induces conformational change of Bak but not Bax, independent of Bid, caspase activation, or ROS generation

Upon their activation, Bak and Bax undergo conformational changes, leading to the exposure of their N-terminal domains (Hsu et al., 1997; Griffiths et al., 1999; Nechushtan et al., 1999). To test whether this process also occurs during GT treatment, wt and $Bak^{-/-} \times Bax^{-/-}$ MEFs were incubated with GT and subsequently analyzed by FACS with conformation-specific antibodies against the N termini of Bak or Bax. As shown in Fig. 3 A, GT was able to readily induce N-terminal epitope exposure in Bak but not in Bax. As expected, no (Bax) or only marginal staining (Bak) was seen with these antibodies in GT-activated $Bak^{-/-} \times Bax^{-/-}$ MEFs. There was no inherent failure of Bax to undergo an N-terminal conformational change in MEFs, as their treatment with staurosporine led to the expected N-terminal opening of Bax (Fig. 3 B; Griffiths et al., 1999; Wei et al., 2001).

To determine the order of events during Bak activation, ROS production, and caspase activation, we incubated GT-treated MEFs with the antioxidants N-acetylcysteine (NAC) or the manganese porphyrin Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP; Faulkner et al., 1994; Day et al., 1997) or the pan-caspase inhibitor ZVAD-fmk (Pardo et al., 2004). NAC and MnTBAP were both effective as antioxidants, as they significantly reduced GT-induced ROS production in MEFs (Fig. 3 C). Moreover, ZVAD-fmk blocked apoptosis induced by the anti(α)-Fas mAb Jo-2 in MBL-2-Fas cells (Fig. 3 C). GT-induced N-terminal opening of Bak was unaffected by any of the three inhibitors (Fig. 3 D), indicating that

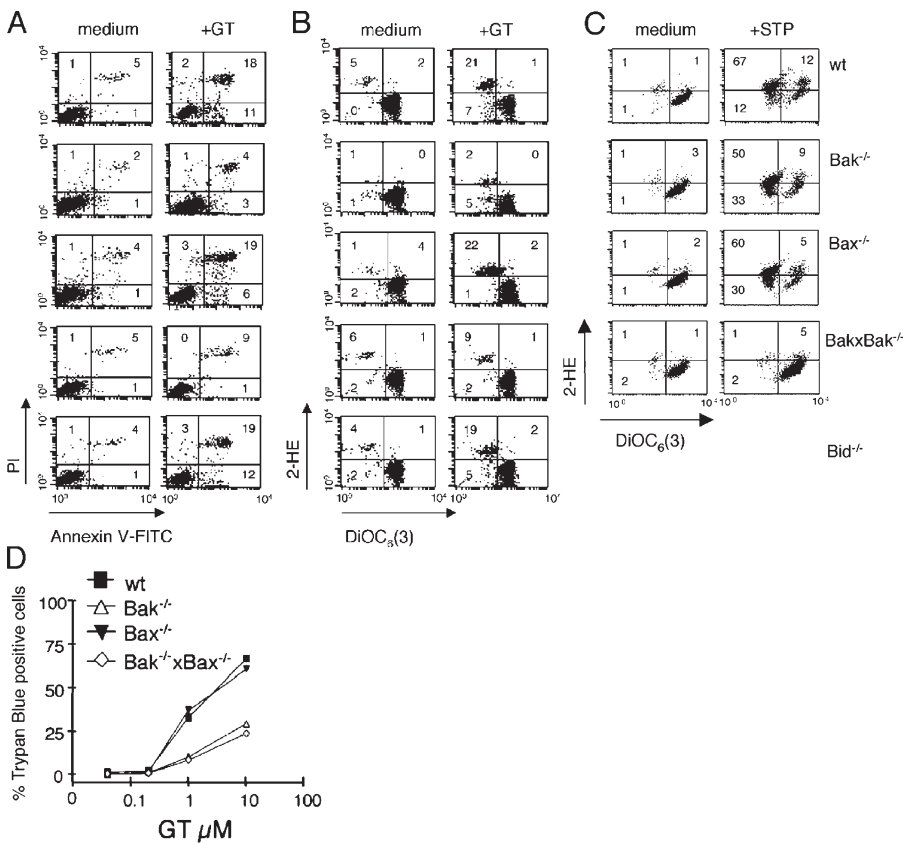


Figure 2. GT-induced apoptosis is Bak dependent. (A and B) wt, $Bak^{-/-}$, $Bax^{-/-}$, $Bak^{-/-} \times Bax^{-/-}$, and $Bid^{-/-}$ MEFs were incubated with or without 1 μ M GT for 4 h and analyzed by FACS for PS exposure (annexin V-FITC) and PI uptake (A) or $\Delta\psi_m$ loss (DiOC₆[3]) and ROS generation (2-HE; B). (C) wt, $Bak^{-/-}$, $Bax^{-/-}$, and $Bak^{-/-} \times Bax^{-/-}$ MEFs were incubated with or without 1 μ M staurosporine (STP) for 12 h and analyzed by FACS, as in B. The same cells were incubated with increasing amounts of GT for 4 h to determine the percentage of cell death (trypan blue exclusion) by microscopic inspection (D). Data shown in A, B, and D are representative of at least four independent experiments with similar outcome.

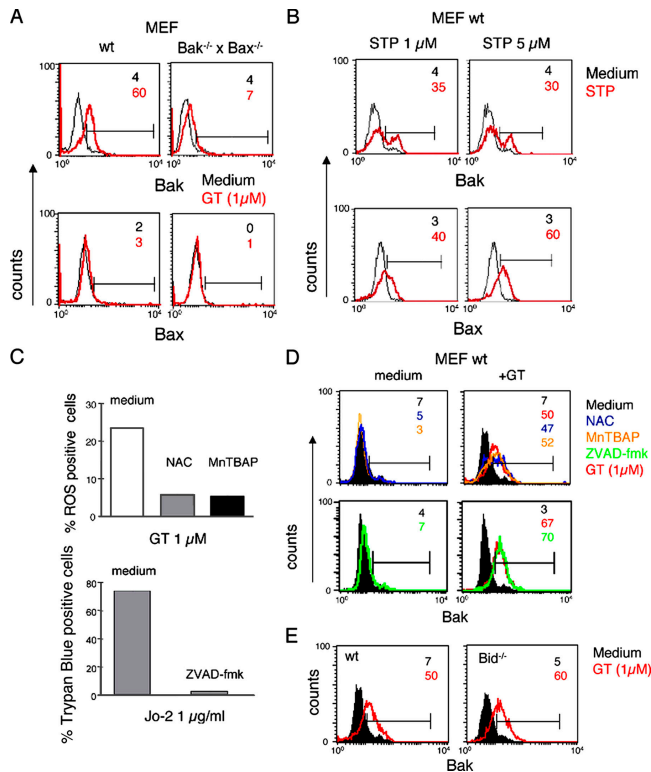


Figure 3. GT induces conformational change of Bak. (A and B) GT induces conformational change of Bak, but not Bax, in MEFs. wt and Bak^{-/-} × Bax^{-/-} MEFs were incubated for 4 h without (black) or with (red) either 1 μM GT (A) or 1 and 5 μM staurosporine (B) and analyzed by FACS for conformational changes of Bak and Bax using mAbs specific for the functionally active N-terminal region in each protein. Numbers given are the percentages of cells positive for active Bak or Bax (indicated by the horizontal bars). (C–E) GT-induced conformational change of Bak is independent of caspase activation, ROS generation, and the presence of Bid. (C) The ROS scavengers NAC and MnTBAP were tested for their efficiency in inhibiting ROS production by FACS analysis (2-HE) of wt MEFs treated with 1 μM GT for 4 h. To test for the caspase-inhibiting potency of ZVAD-fmk, MBL-2-Fas cells were treated with 1 μg/ml of the α-Fas antibody Jo-2 in the absence and presence of 100 μM ZVAD-fmk, and cell death was monitored by trypan blue exclusion. (D) wt MEFs were incubated with (red) or without (black) 1 μM GT for 4 h in the presence or absence of 100 μM ZVAD-fmk (green), 15 mM NAC, or 200 μM MnTBAP. The cells were analyzed by FACS for conformational changes of Bak as under A. (E) GT-induced conformational change of Bak is independent of Bid. wt and Bid^{-/-} MEFs were treated with GT and FACS analyzed for conformational changes of Bak as in A. Numbers given are the percentages of cells positive for active Bak (indicated by the horizontal bars). Data shown are representative of at least three independent experiments with similar outcome.

ROS production and caspase activation occur downstream of Bak activation. Furthermore, as similar activation of Bak was seen in GT-treated wt and Bid^{-/-} MEFs, GT-mediated conformational change of Bak is also independent of Bid (Fig. 3 E).

GT-induced mitochondrial and membrane damage depends on ROS production

Although the association of ROS generation with GT- or CTL-mediated apoptosis is well documented (Kweon et al., 2003; Pardo et al., 2004), the contribution of ROS to cell death is still controversial (Chandra et al., 2000; Danial and Korsmeyer, 2004). We therefore tested the effect of NAC and MnTBAP on GT-induced plasma and mitochondrial membrane integrity and cell death.

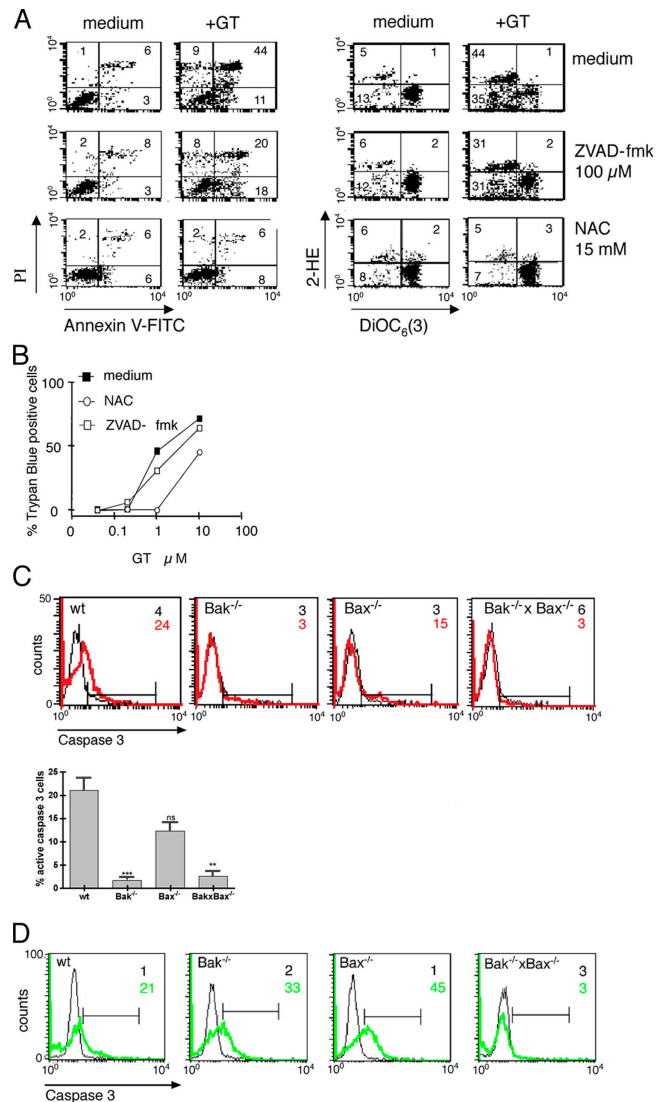


Figure 4. GT-induced mitochondrial membrane perturbation and apoptosis are dependent on ROS generation, whereas only the latter partially depends on caspases. (A) wt MEFs were incubated with or without 1 μM GT for 4 h in the presence or absence of 100 μM of the pan-caspase inhibitor ZVAD-fmk (green) or 15 mM of the ROS scavenger NAC and analyzed by FACS for PS exposure (annexin V-FITC)/PI uptake and Δψ_m loss (DiOC₆[3])/ROS generation (2-HE). (B) wt MEFs were incubated with increasing amounts of GT for 4 h in the presence or absence of ZVAD-fmk or NAC, and the percentage of cell death (trypan blue exclusion) was determined by microscopic inspection. (C) wt, Bak^{-/-}, Bax^{-/-}, and Bak^{-/-} × Bax^{-/-} MEFs were incubated with (red) or without (black) 1 μM GT for 4 h and analyzed by FACS for the activation of caspase-3, using an α-caspase-3 mAb (FITC labeled) against the active form of the enzyme. A representative FACS analysis represented as histogram (C, bottom) and a graph showing the mean ± SEM from four independent experiments (right) is shown. ns, P = 0.1730; **, P = 0.0002; ***, P = 0.0012. Analyzed by two-tailed unpaired *t* test comparing wt versus knockouts. (D) wt, Bak^{-/-}, Bax^{-/-}, and Bak^{-/-} × Bax^{-/-} MEFs were incubated with (green) or without (black) 1 μM staurosporine for 4 h and analyzed by FACS for the activation of caspase-3, as in C.

As shown in Fig. 4 A, the addition of NAC to wt MEFs at a concentration known to inhibit ROS generation (Fig. 3 C; Pardo et al., 2004) abrogated GT-induced PS exposure and plasma membrane permeability, as well as the reduction of the mitochondrial Δψ_m. Moreover, at 1 μM GT, NAC totally inhibited cell

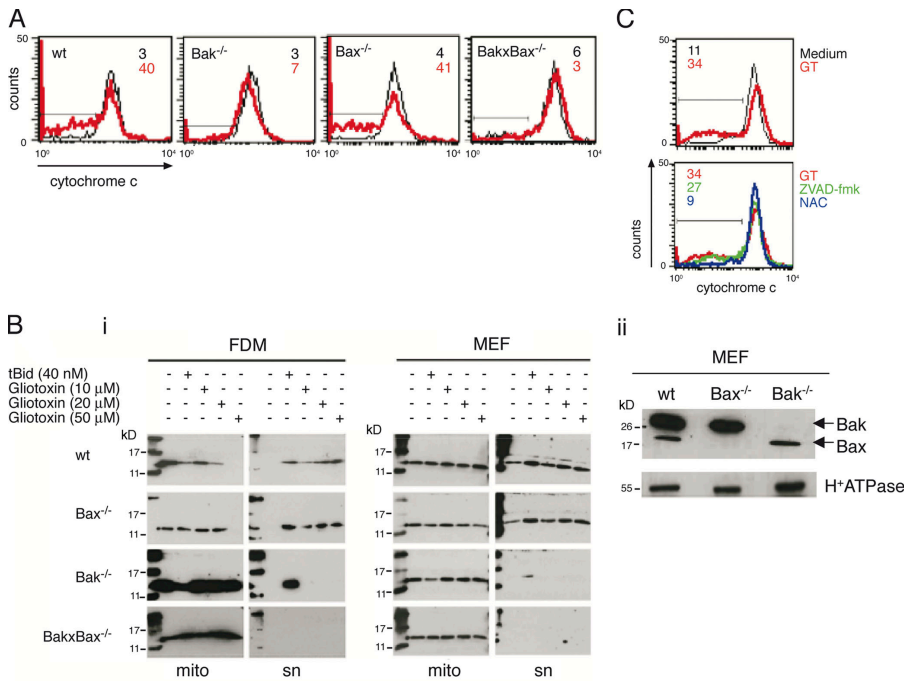


Figure 5. GT-induced cytochrome *c* release from mitochondria is dependent on Bak and the ROS generation. (A) wt, Bak^{-/-}, Bax^{-/-}, and Bak^{-/-} × Bax^{-/-} MEFs were incubated with (red) or without (black) 1 μM GT for 4 h and analyzed by FACS for mitochondrial release of cytochrome *c* using an α-cytochrome *c* mAb followed by a FITC-labeled secondary antibody. Numbers depicted are the percentages of cells negative for cytochrome *c* (indicated by horizontal bars). (B) Mitochondria isolated from wt, Bax^{-/-}, Bak^{-/-}, and Bak^{-/-} × Bax^{-/-} FDMs (left) or MEFs (right) were treated with 10, 20, or 50 μM of GT or 40 nM of tBid for 4 h in vitro. After centrifugation, cytochrome *c* was measured in mitochondria and the supernatant by α-cytochrome *c* Western blotting (i). In parallel, the presence of Bak and Bax was analyzed in MEF wt, Bax^{-/-}, and Bak^{-/-} by simultaneous α-Bax and α-Bak immunoblotting of the mitochondrial preparations. As control, an antibody against the α subunit of the H⁺-ATPase was used (ii). (C) wt MEF cells were incubated with (red) or without (black) 1 μM GT for 4 h in the presence or absence of 100 μM ZVAD-fmk (green) or 15 mM NAC (blue) and analyzed by FACS for mitochondrial release of cytochrome *c* as in A. Data shown are representatives of at least three independent experiments with similar outcome.

death (according to the absence of trypan blue staining; Fig. 4 B). Similar results were obtained with MnTBAP, indicating that the effect of NAC was due to its antioxidant properties (unpublished data). These data reveal that ROS generation is crucial for GT-induced changes of plasma membrane permeability, the mitochondrial $\Delta\psi_m$, and cell death.

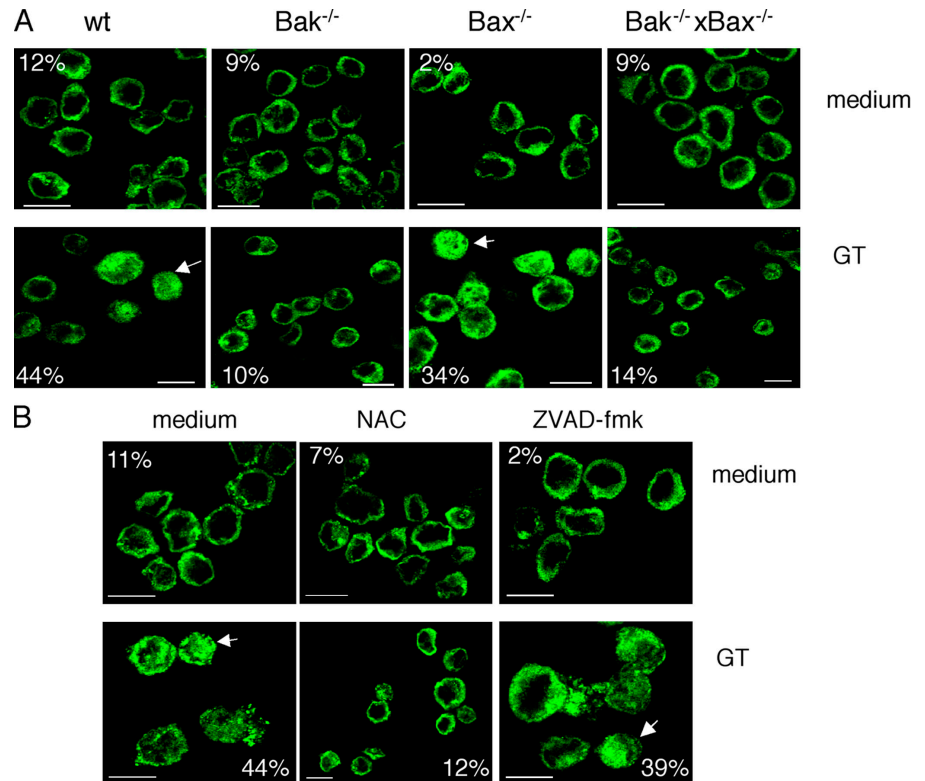
GT-induced caspase-3 activation depends on Bak activation

To determine the role of caspases in the GT-induced reduction of the mitochondrial $\Delta\psi_m$ and apoptosis, we tested whether GT could induce cell death and changes in mitochondrial $\Delta\psi_m$ in the presence of the broad spectrum caspase inhibitor ZVAD-fmk. 100 μM ZVAD-fmk did not prevent loss of the mitochondrial $\Delta\psi_m$ in GT-treated cells, indicating that caspase activation was not needed for this event. However, ZVAD-fmk partially reduced GT-induced annexin V/PI staining (Fig. 4 A) but did not affect cell death (Fig. 4 B). These data also exclude a possible role of death receptor-mediated cell death, which is completely blocked by caspase inhibitors (Fig. 3 C; Longthorne and Williams, 1997). To test whether the major downstream effector caspase, caspase-3, was involved in these processes, and whether activation of this caspase was dependent on Bak, we performed a FACS analysis of wt and ^{-/-} MEFs using an α-caspase-3 mAb specific for the processed active form of caspase-3. As shown in Fig. 4 C, caspase-3 was significantly activated in GT-treated wt and Bax^{-/-} but not in Bak^{-/-} and Bak^{-/-} × Bax^{-/-} MEFs. In contrast, staurosporine induced caspase-3 activation in Bak^{-/-} and Bax^{-/-} but not in Bak^{-/-} × Bax^{-/-} MEFs (Fig. 4 D), again showing that both Bak and Bax can undergo conformational activation upon appropriate stimulus (Wei et al., 2001).

GT-induced release of cytochrome *c* depends on Bak activation and ROS production

The activation of Bak and the subsequent production of ROS and the reduction of mitochondrial $\Delta\psi_m$ in response to GT indicate increased mitochondrial membrane permeability, leading to the release of apoptogenic factors such as cytochrome *c* and AIF. Although cytochrome *c* activates caspase-3 via the apoptosome (Li et al., 1997), AIF translocates to the nucleus and contributes to DNA fragmentation in a caspase-independent manner (Susin et al., 1999; Pardo et al., 2001). The release of both cytochrome *c* and AIF from mitochondria is absolutely dependent on activation of Bax, Bak, or both (Lindsten et al., 2000; Wei et al., 2001). To test whether GT-induced cytochrome *c* release selectively required Bak, wt, Bak^{-/-}, Bax^{-/-}, or Bak^{-/-} × Bax^{-/-} MEFs were incubated with GT and mitochondrial cytochrome *c* was quantitatively measured by FACS analysis (Fig. 5 A). In addition, AIF release was monitored by α-AIF immunofluorescence (Fig. 6 A). As shown in Fig. 5 A, mitochondrial cytochrome *c* was reduced in GT-treated wt and Bax^{-/-} MEFs but retained in Bak^{-/-} or Bak^{-/-} × Bax^{-/-} MEFs. In addition, a high portion of GT-treated wt and Bax^{-/-} MEFs displayed cytosolic localization and nuclear translocation of AIF, whereas Bak^{-/-} and Bak^{-/-} × Bax^{-/-} MEFs retained most of the AIF in the mitochondria (Fig. 6 A). These data confirm that GT induced mitochondrial membrane permeability; i.e., cytochrome *c* and AIF release occurs by a process selectively involving Bak. To test whether GT could directly act on mitochondrial Bak without the requirement of any cytosolic factors, such as, for example, a particular BH3-only protein, we compared GT-induced cytochrome *c* release on isolated mitochondria from wt MEFs with those of knockout MEFs and factor-dependent myeloids (FDMs).

Figure 6. **GT-induced AIF translocation to the nucleus is dependent on Bak and the generation of ROS.** (A) wt, Bak^{-/-}, Bax^{-/-}, and Bak^{-/-} × Bax^{-/-} MEF cells were incubated with or without 1 μM GT for 4 h and analyzed by confocal microscopy for nuclear translocation of AIF using a specific α-AIF rabbit antibody. Numbers depicted are the percentages of cells with nuclear AIF. (B) Alternatively, wt MEFs were incubated with or without 1 μM of GT for 4 h in the presence or absence of 100 μM ZVAD-fmk or 15 mM NAC and analyzed for nuclear translocation of AIF as described above. Data are representative of two independent experiments. Bars, 10 μm.



As shown in Fig. 5 B i, 10–50 μM GT caused cytochrome *c* release from isolated wt mitochondria in a dose-dependent manner. The requirement for much higher concentrations of GT to induce cytochrome *c* release in isolated mitochondria as compared with intact cells is in line with a previous study in which GT-mediated calcium release was analyzed (Schweizer and Richter, 1994). In fact, it is known that levels of GT determined intracellularly do exceed those originally applied in solution by up to 1,500-fold (Waring et al., 1994; Bernardo et al., 2003). The release of cytochrome *c* was as efficient as that induced by recombinant tBid, a known inducer of mitochondrial membrane permeability via Bak/Bax (Wei et al., 2000, 2001) and significantly greater than the background release of cytochrome *c* observed in mock-treated mitochondrial preparations. Strikingly, although mitochondria from Bax^{-/-} showed similar GT-induced cytochrome *c* release as wt mitochondria, mitochondria from Bak^{-/-} or Bak^{-/-} × Bax^{-/-} did not, irrespective of whether they were derived from MEFs or FDMs. This suggests that GT does not need cytosolic factors such as tBid or caspases to elicit cytochrome *c* release but may act directly on mitochondrial Bak or some as-yet-unknown mitochondrial protein that activates Bak with a potency similar to tBid. We excluded the possibility that the resistance of GT-facilitated cytochrome *c* released could be due to the absence of Bax on mitochondria, as appreciable amounts of this protein were detected on isolated, washed mitochondria from both wt and Bak^{-/-} FDMs (Fig. 5 B ii). Moreover, tBid was capable of inducing cytochrome *c* release from mitochondria isolated from both Bak^{-/-} FDMs and MEFs. This would not have been possible if they had no Bax.

Consistent with a direct action of GT on mitochondrial membrane permeability, GT-induced cytochrome *c* and AIF release was not blocked by ZVAD in wt MEFs (Fig. 5 C and Fig. 6 B). Most important, however, both cytochrome *c* and AIF release from mitochondria was greatly diminished by NAC (Fig. 5 C and Fig. 6 B). This suggests that GT-induced ROS production is crucial for effective release of apoptogenic factors from mitochondria but does not exclude the possibility that NAC has some other mitochondrial membrane stabilizing activity.

Bak-deficient mice are resistant to IA

To determine whether the selective activation of Bak during GT-mediated cell death as seen in our in vitro analysis is of biological relevance and pathophysiological significance, we monitored the mortality of immunosuppressed (hydrocortisone-treated) wt (C57BL/6) and Bak ko (Bak^{-/-}) mice subsequently infected with a GT-producing *A. fumigatus* strain. The presence of GT synthesis by this fungal strain was verified by HPLC analysis of culture SN (Fig. 7 B). Fig. 7 A shows that 5 out of 6 wt mice died within 2 wk after intranasal infection, and only 1 out of 6 Bak^{-/-} mice succumbed over the same time period ($P < 0.015$). These in vivo data correlate with our in vitro findings and show for the first time that Bak is a host susceptibility factor for *A. fumigatus* virulence in mice, probably because of its direct activation by GT.

Discussion

Although GT has long been proposed to constitute a virulence factor in IA (Eichner and Mullbacher, 1984; Mullbacher

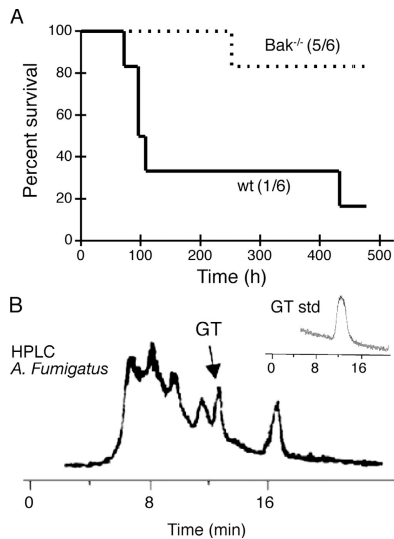


Figure 7. Deletion of Bak results in resistance to mice of *A. fumigatus*. (A) *Bak*^{-/-} mice are resistant to *A. fumigatus* infection. Female wt C57BL/6 or *Bak*^{-/-} mice (6 mice per group) were immunosuppressed by subcutaneous injection of 2 mg of hydrocortisone in PBS/0.1% Tween-20 on days -4, -2, 0, 2, and 4 of infection. On day 0, recipients were inoculated intranasally with 5×10^6 *A. fumigatus* B5233 in 20 μ l of PBS or with 20 μ l PBS alone (control), and morbidity and mortality was monitored during the time. Control mice survive without any symptoms of infection. Survival was significantly different ($P < 0.015$), as analyzed by the Kaplan-Meier survival test. (B) *A. fumigatus* B5233 is able to produce GT. 10^7 *A. fumigatus* spores were inoculated in 100 ml of RPMI and grown for 48 h at 37°C and 0.5% CO₂, and the presence of GT in the SN (black) was assessed by HPLC, using a GT standard (inset) as described in Materials and methods.

and Eichner, 1984), most probably by suppressing immune responses via induction of mammalian cell apoptosis (Waring et al., 1988b; Sutton et al., 1994), the molecular mechanisms underlying the putative in vitro and in vivo processes have not been elucidated. Here, we present evidence that Bak, but not Bax, is a key host factor in GT-mediated cell death in vitro. We used MEFs and FDMs deficient in the Bcl-2 family members Bak and/or Bax or their activator Bid (Wei et al., 2000) and isolated mitochondria from these cells to show that GT-mediated activation of Bak occurs independently of Bid or other cytosolic factors. Once activated, Bak triggers the generation of ROS, which is crucial for effective mitochondrial membrane pore formation, including the release of cytochrome *c* and AIF, and ultimate cell death. The additional finding that the virulence of GT-producing *A. fumigatus* was significantly decreased in *Bak*^{-/-} over wt mice strongly implicates that GT is an important modulator in mammalian host defense and that Bak is a prominent host susceptibility factor. The interrelation of GT and Bak in *A. fumigatus* pathology is also supported by the recent finding that an *A. fumigatus* mutant lacking GT expresses a drastically reduced virulence (unpublished data).

At present, it is unclear how GT activates Bak. One mechanism by which GT may activate Bak is by breaking up inhibitory complexes between Bak and Bcl-2-like prosurvival factors (Sattler et al., 1997; Cuconati et al., 2003; Willis et al., 2005; Ekert et al., 2006) or VDAC2 (Cheng et al., 2003) on the mitochondrial membrane. This could be by forming transient

disulphide bonds between the reactive disulphide bond in GT and individual cysteine residues in Bak or its binding partners, leading to the release of active Bak. Three-dimensional structures of Bak-Bcl-2 or Bak-VDAC2 complexes have not yet been determined, so we do not know if any cysteine residues are involved in these interactions. Moreover, we do not have any experimental evidence that GT directly binds Bak, Bcl-2-like proteins, or VDAC2. However, our data obtained with isolated mitochondria favor an interpretation that GT-facilitated activation of Bak occurs by direct interaction with antiapoptotic Bcl-2 family members or other mitochondrial membrane-associated constituents. The former possibility is supported by the observation that protection against GT-mediated monocyte apoptosis by agonists of nerve growth factor receptors is associated with the up-regulation of Bcl-2 and Bcl-x_L (la Sala et al., 2000).

The finding that GT specifically acts through Bak and not Bax is intriguing. Although both proteins are supposed to exert the same pore-forming activity on mitochondria (Kuwana et al., 2005), they are activated differently. There is increasing evidence for selective Bax- or Bak-specific apoptosis, depending on the cell type and the apoptotic stimuli (Lindenboim et al., 2005; Wendt et al., 2005). Thus, the activation mechanism of Bax and Bak may be distinct, although both ultimately oligomerize and form pores in the outer mitochondrial membrane. In this respect, GT may be unable to interact with Bax and/or to release any of its inhibitory components. Moreover, an interaction of GT with Bcl-2 or Bcl-x_L would not affect Bax because it is not sequestered by these proteins in healthy cells. This would explain why GT induces conformational activation of Bak but not of Bax.

The findings that the mitochondrial protein VDAC2 associates with and inhibits Bak in healthy mitochondria (Cheng et al., 2003) and that in monocytes, Bak but not Bax is part of the VDAC channel (unpublished data) suggest VDAC2's involvement in GT-mediated cell death. VDAC2 is one of three mammalian isoforms of VDAC proteins (VDAC1, -2, and -3), which constitute the major pathway for metabolic exchange across the outer mitochondrial membrane (Sampson et al., 1997; Wu et al., 1999; Xu et al., 1999). Together with cyclophilin D and adenine nucleotide transporter (ANT), VDAC forms the mitochondrial permeability transition pore (MPTP), involved in cell apoptosis and/or necrosis (Crompton, 1999; Zheng et al., 2004). How the function of MPTP is regulated by members of the BH3 family is still highly controversial (Marzo et al., 1998; Shimizu et al., 1999; Vander Heiden et al., 1999). One could postulate that GT somehow modulates the VDAC complex, leading to the liberation of Bak, a subsequent increase of mitochondrial membrane permeability and hence a Bak-dependent cytochrome *c* release and cell death. The contribution of the MPTP in the latter process is further supported by the findings that GT-induced apoptosis of activated hepatic stellate cells is associated with a specific thiol redox-dependent interaction with MPTP component ANT (Orr et al., 2004) and that cyclosporin A, an inhibitor of cyclophilin D and mitochondrial pore opening (Crompton et al., 1988), affected mitochondrial depolarization and ROS production (Kweon et al., 2003; unpublished data). Most notable, the data suggested that oxidative cross-linking of

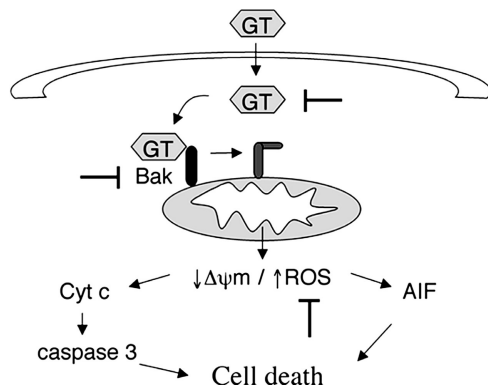


Figure 8. Proposed mechanism for GT-induced cell death and aspergillosis treatment. After production by *A. fumigatus*, GT would enter cells by a redox-dependent mechanism and directly induce a conformational change in Bak, leading to mitochondrial depolarization and ROS production. ROS production then triggers the mitochondrial release of apoptogenic proteins such as cytochrome *c* (cyt *c*) and AIF. In this way, both caspase-dependent and -independent processes would be launched to induce cell death. By blocking either GT or Bak conformational change or even ROS production, GT-induced cell death could be prevented and the damage exerted by *A. fumigatus* attenuated.

two matrix-facing cysteine residues on the ANT (Cys⁵⁶ and Cys¹⁵⁹) plays a key role in regulating the MPTP (Halestrap et al., 2002). However, more detailed studies, including MPTP inhibitors such as bongkreic acid or cyclosporins A, are required to dissect the role of the VDAC-ANT complex in GT-mediated and Bak-dependent cell death.

Our data further show that GT-induced production of ROS is mandatory for cell death. The sequence of events leading to ROS production by GT was revealed by analyzing the various proapoptotic processes in the presence of inhibitors for ROS and for caspases, including NAC, MnTBAP, and ZVAD-fmk, respectively (Fig. 8). Accordingly, activation of Bak precedes the generation of ROS, which then facilitate the release of cytochrome *c* and AIF from mitochondria, leading to caspase activation as well as mitochondria- and caspase-independent events to mediate cell death. As to the source of ROS, it is possible that they are generated from a perturbation of mitochondrial respiration that is due to Bak-mediated pore formation and/or activation of MPTP. Why ROS are, at least partially, required for cytochrome *c* release is unclear, although it has been shown that ROS generation is crucial for cytochrome *c* release under different apoptotic stimuli (Petrosillo et al., 2001; Orrenius and Zhivotovsky, 2005; Santamaria et al., 2006).

The putative relevance of GT-mediated apoptosis for the parasitized vertebrate host was analyzed by comparing the course of *A. fumigatus* infection in wt and Bak^{-/-} mice. The significantly decreased virulence of the pathogen observed in Bak^{-/-} as compared with wt mice, as revealed by the differential kinetics of mortality, supports the following assumptions: GT is released during *A. fumigatus* infection, as suggested before (Eichner and Mullbacher, 1984; Lewis et al., 2005), and induces apoptosis in multiple target cells, most probably via Bak activation. This process subsequently leads to an accelerated colonization of target organs by breaching physical barriers, such as lung and renal epithelial cells, and establishes

an immunosuppressed state of the host. Although the present data do not formally prove a cause-effect relationship between GT, Bak activation, and *A. fumigatus* pathogenicity (virulence), the previous (Eichner and Mullbacher, 1984; Mullbacher and Eichner, 1984) and present assumption that GT is a virulence factor of *A. fumigatus* is supported by a recent report (Bok et al., 2005) and our own unpublished data. These results have shown that low levels of pulmonary GT observed with an *A. fumigatus* mutant defective in LaeA, a global regulator of secondary metabolism, is associated with impaired virulence of the pathogen. Furthermore, by using a recently generated *glp* gene knockout mutant of *A. fumigatus* lacking GT, we found that this mutant is much less virulent in mice than the wt strain and that cell culture supernatants were unable to induce cell death (unpublished data).

Based on the sequence of intracellular events occurring during GT-induced apoptosis (Fig. 8), we conclude that GT is a critical virulence factor in *A. fumigatus*. This is supported by the fact that GT is one of the most abundant secondary metabolites produced by the fungus (Taylor, 1971) and that Bak^{-/-} mice are more resistant to infection by *A. fumigatus*. The distinct potential of GT to activate Bak, but not Bax, may be of relevance for the development of anti-IA drugs that selectively block cell death pathways via Bak and, at the same time, spare the residual proapoptotic proteins relevant for the control of the pathogen by the host's immune system.

Materials and methods

Cell culture and reagents

SV40 transformed MEFs (Wei et al., 2001) and MBL-2-Fas cells were cultured in MEM supplemented with 10% FCS and 2-mercaptoethanol (10⁻⁵ M) at 37°C and 7% CO₂. The IL-3-dependent (FDM) cell lines were generated by coculturing embryonic day 14.4 fetal liver single-cell suspensions with fibroblasts expressing a HoxB8 retrovirus in the presence of high IL-3 concentrations, as previously described (Ekert et al., 2004). Bak^{-/-} mice were obtained from C. Thompson (Harvard Medical School, Boston, MA), backcrossed for nine generations to ensure a "pure" C57BL/6 genetic background, and intercrossed with Bax^{-/-} C57BL/6 mice to obtain Bax^{-/-} × Bak^{-/-} mice (provided by D. Huang, The Walter and Eliza Hall Institute, Melbourne, Australia) as described previously (Willis et al., 2005). The cell lines were cultured in MEM with Earle's salts and L-glutamine. For Western blot analysis of isolated mitochondria, antibodies to H⁺-ATPase (Invitrogen) were used as controls.

GT was purified from *Penicillium terlikowskii* as described previously (Waring et al., 1988b). The purity of this preparation was analyzed by TLC and HPLC showing the same quality as commercial GT. For apoptosis induction, 2 × 10⁵ cells/ml MEFs were incubated with the indicated concentration of GT or staurosporine (Sigma-Aldrich) for 4 h, and apoptosis assays were performed as described in the following paragraphs. In some cases, the general caspase inhibitor Ac-ZVAD-fmk (Bachem) or the ROS scavengers NAC (Sigma-Aldrich) or MnTBAP (Calbiochem) were added as described previously (Pardo et al., 2004). To test the inhibitory potency of Ac-ZVAD-fmk, MBL-2-Fas cells were incubated with 1 μg/ml α-Fas mAb Jo-2 for 24 h in the presence or absence of 100 μM of the caspase inhibitor, and cell death was analyzed by trypan blue exclusion. Nuclei were stained with 10 μg/ml Hoechst 33342 (Invitrogen).

Nuclear, cell membrane, and mitochondrial membrane perturbation

PS exposure and PI uptake was analyzed by FACS or fluorescence microscopy as described previously (Pardo et al., 2004) using the annexin V-FITC kit from BD Biosciences. Δψ_m was measured with the fluorescent probe 3,3'-dihexyloxacarbocyanine iodide (DiOC₆[3]; Invitrogen) and ROS generation with 2-hydroxyethidine (2-HE; Invitrogen) as described previously (Pardo et al., 2004). Nuclear morphology was analyzed by fluorescence microscopy with Hoechst 33342. For that, cells were fixed with

1% PFA and mounted on a drop of Fluoromount-G (Southern Biotechnology Associates, Inc.) containing 10 μ g/ml Hoechst 33342, and images were taken at room temperature using a microscope (Axioskop 10; Carl Zeiss Microimaging, Inc.), an analysis camera (AxioCam; Carl Zeiss Microimaging, Inc.), and Vision 3.1.0.0 software (Carl Zeiss Microimaging, Inc.). The objective used was a PlanNeofluor (Carl Zeiss Microimaging, Inc.), with a magnification of 40 and a NA of 0.75. Photoshop CS2 (Adobe) was used for minor adjustments to contrast and image overlay. In some cases, membrane perturbation was also analyzed by fluorescence microscopy by staining the cells with the annexin V-FITC kit from BD Biosciences. Images were taken in the same conditions as described above, but cells were mounted on annexin binding buffer (BD Biosciences).

Caspase-3 activation

Cells were fixed with 2.5% PFA, incubated with a mAb FITC-labeled against the active form of caspase-3 (clone C92605; BD Biosciences), and analyzed by FACS as described previously (Pardo et al., 2004).

Cytochrome c release and nuclear translocation of AIF on cells

Cytochrome c release was quantified by FACS analysis as recently described (Waterhouse and Trapani, 2003). In brief, 10^6 MEFs were mildly permeabilized with 25 μ g/ml digitonin plus 100 mM KCl on ice for 5 min. This led to the cellular loss of cytosolic cytochrome c. Cells were washed once with cold PBS, fixed in 4% PFA, permeabilized with 0.05% saponin and 3% BSA, and incubated with the α -cytochrome c mAb 6H2.B4 (BD Biosciences) or mouse IgG isotype control (Jackson ImmunoResearch Laboratories) followed by α -mouse-FITC secondary antibody (Jackson ImmunoResearch Laboratories). The cells were resuspended in 100 μ l PFA in PBS and analyzed by FACS with a FACScan (BD Biosciences) and CellQuest software (BD Biosciences). For the analysis of the nuclear translocation of AIF, cells were fixed, mounted on poly-L-lysine cover slides, stained with a rabbit polyclonal α -AIF antibody (Sigma-Aldrich) followed by the secondary goat α -rabbit antibody labeled with Alexa 488 as described previously (Pardo et al., 2001), and mounted on a drop of Fluoromount-G. Afterward, the cells were analyzed by confocal microscopy. Fluorescence images were taken at room temperature on a confocal microscope (TCS SP2; Leica) using a 40 \times objective (HCX PL APO CS; Leica), NA 1.25, immersion oil, and confocal software (version 2.61; Leica). Photoshop CS2 was used for minor adjustments to contrast.

Cytochrome c release from isolated mitochondria

8×10^7 MEFs or FDMs were centrifuged and washed once in PBS. The cell pellets were resuspended in 500 μ l MSH buffer (210 mM mannitol, 70 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.5, 100 μ M PMSF, 400 ng/ml pepstatin, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 5 μ g/ml cytochalasin B). The resuspended cell pellet was incubated on ice for 15 min before the cells were broken by passing 25 times through a 23-gauge needle. The lysate was centrifuged at 500 g for 5 min to remove cell debris and nuclei. A crude mitochondrial pellet was then obtained by centrifugation at 10,000 g for 15 min and resuspended in MSH buffer. The isolated mitochondria were incubated with different concentrations of GT (10, 20, and 50 μ M) or 40 nM of recombinant tBid (provided by J.C. Martinou, University of Geneva, Geneva, Switzerland) as a positive control at 37°C for 4 h. After incubation, the mitochondria were pelleted, and both pellet and supernatant were tested for cytochrome c release by SDS-PAGE.

Conformational change of Bax and Bak

MEFs were fixed in 4% PFA, permeabilized with 0.1% saponin in PBS/5% FCS, and incubated with 2 μ g/ml rabbit polyclonal α -Bak (NT; Upstate Biotechnology), 5 μ g/ml rabbit polyclonal α -Bax (NT; Upstate Biotechnology), or 5 μ g/ml rabbit purified IgG (control). After two washes with 0.1% saponin in PBS, the cells were incubated with α -rabbit-FITC antibody in 0.1% saponin/PBS/5% FCS, washed twice in 0.1% saponin/PBS, resuspended in 1% PFA/PBS, and analyzed by FACS with a FACScan and CellQuest software. The amount of Bax and Bak on isolated mitochondria from wt, Bak^{-/-}, Bax^{-/-}, and Bak^{-/-} \times Bax^{-/-} FDMs was determined by washing the centrifuged mitochondria twice in large amounts MSH buffer (to eliminate cytosolic contamination) followed by lysing the mitochondria in SDS sample buffer and analysis by α -Bax (Bax-NT) and α -Bak (Bak-NT) Western blotting on the same gel. As mitochondrial marker and loading control, an antibody against the FOF1 ATPase was used.

In vivo invasive aspergillosis model and GT analysis on culture supernatants

Female mice [C57BL/6, Bak^{-/-} [Jackson ImmunoResearch Laboratories; C57BL/6.129, six times backcrossed in C57BL/6], or 129/Sv] were

immunosuppressed by subcutaneous injection of 3 mg (112 mg/kg) of hydrocortisone (Sigma-Aldrich) diluted in 200 μ l of PBS/0.1% Tween 20 on days -4, -2, 0, 2, and 4, as described previously (Tang et al., 1993). On day 0, mice (6 per group) were infected intranasally with 5×10^6 *A. fumigatus* B5233 conidia in 20 μ l of PBS or with PBS alone. Disease development was analyzed by morbidity/mortality of the mice after infection. There was no difference in the sensitivity of C57BL/6 or 129/Sv mice to *A. fumigatus* infection, and all infected recipients of both mouse strains died during the first week after infection. GT presence on *A. fumigatus* B5233 culture supernatants was analyzed after 48 h by HPLC as described previously (Belkacemi et al., 1999).

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