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Capsular polysaccharide induction of apoptosis by intrinsic and extrinsic mechanisms

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Summary

A purified microbial capsular polysaccharide of *Cryptococcus neoformans,* glucuronoxylomannan (GXM), induces Fas ligand (FasL) upregulation on macrophages and, as a consequence, apoptosis of lymphocytes. The mechanisms that lead to lymphocyte apoptosis in both *in vitro* and *in vivo* systems were investigated by cytofluorimetric analysis and Western blotting experiments. Caspase 8 cleaves caspase 3 in two different pathways: directly as well as indirectly by activation of Bcl-2 interacting domain, which initiates caspase 9 cleavage. Therefore, the caspase 8 and caspase 9 pathways cooperate in an amplification loop for efficient cell death, and noteworthily we provide evidence that they are both activated in one single cell. Furthermore, both activation of GXM-mediated caspase 8 and apoptosis were also found in *in vivo* systems in an experimental model of murine candidiasis. Collectively, our data show that GXM-induced apoptosis involves, in a single cell, a cross-talk between extrinsic and intrinsic pathways. Such a finding offers opportunities for the therapeutic usage of this polysaccharide in appropriate clinical settings for taming T-cell responses.

Introduction

Apoptosis is a fundamental biological mechanism used by nearly all types of tissues and cells. It is essential to embryogenesis, tissue renewal, receptor repertoire selection and immune regulation. The occurrence of either exacerbated or deficient apoptosis is associated with disease.

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The Fas-FasL system plays an important role in the maintenance of T-cell tolerance and in prevention of autoimmune disease (Askenasy *et al*., 2005), as shown by the finding that mutations of Fas or FasL genes lead to autoimmune disease in lpr/lpr and gld/gld mice respectively (Watanabe-Fukunaga *et al*., 1992; Nagata and Suda, 1995).

Fas is a type I transmembrane receptor with a single transmembrane domain of 17 amino acids, an N-terminal cysteine-rich extracellular domain, and a C-terminal cytoplasmatic domain of 145 amino acids. The cytoplasmatic portion of Fas contains a domain of about 85 amino acids termed 'death domain', which plays a crucial role in transmitting the death signal from the cell surface to intracellular pathways. FasL, a type II transmembrane protein, is its ligand (Nagata, 1999). The binding of FasL with its receptor induces apoptosis of the target cell. Fas is constitutively expressed in many different tissues, whereas the constitutive expression of FasL is restricted to cells present in immune privileged organs, such as Sertoli cells of the testis and epithelial cells of the eye (Griffith *et al*., 1995). Immune privilege is, at least in part, caused by induction of apoptosis on recruited Fas-expressing inflammatory cells (Griffith *et al*., 1995). In addition, FasL expression can be induced in a strictly controlled manner in lymphoid and non-lymphoid cells (Kavurma and Khachigian, 2003).

Apoptosis is initiated when Fas is engaged by FasL trimers to form the death-inducing signalling complex (DISC), and it is orchestrated by the action of a set of proteases in the cell, called caspases (cysteine proteinases) (Krammer, 2000). Caspase 8 is an essential component of certain death receptor pathways, and caspase 8 null embryonic fibroblasts are resistant to the cytotoxic effects of FasL, TNF and TRAIL. Upon activation of caspase 8 within the DISC, the death signal is propagated by two alternative mechanisms, depending on the cell type. In type I cells, the extrinsic pathway involves caspase 8 activation, which is capable of activating downstream effector caspases such as caspases 3, 6 and 7, leading to apoptosis. Conversely, in type II cells, in which DISC formation is significantly reduced, the apoptotic signal has to be amplified by the intrinsic mitochondrial apoptosis pathway. In these cells, caspase 8 activation results in the proteolysis of Bcl-2 interacting domain (BID), which then translocates to mitochondria with

induction of mitochondrial pathways of apoptosis (Creagh *et al*., 2003). Upon activation, mitochondria release pro-apoptotic molecules such as cytochrome *c* and Smac/DIABLO. Together with the apoptotic protease-activating factor 1 (Apaf-1) and procaspase 9 in the cytoplasm, these molecules form the apoptosome. Caspase 9 activates further downstream caspases, and the end result is apoptosis (Green, 2003).

Macrophage-induced apoptosis is emerging as a macrophage effector function. Activated macrophages express FasL and are able to induce apoptosis of T cells (Oyaizu *et al*., 1997). In a previous study, we demonstrated that macrophages loaded with glucuronoxylomannan (GXM), the capsular polysaccharide of *Cryptococcus neoformans*, showed an upregulation of FasL surface expression and were able to induce apoptosis in activated $CD3^+$ T cells. GXM is the most important virulence factor of *C. neoformans* and is found bound to the fungal cell in the form of a capsule or shed in soluble form during *in vivo* and *in vitro* growth. GXM interacts with several effector cells such as monocytes, macrophages and dendritic cells, and it transmits multiple immunoinhibitory signals via its engagement of FcγRIIB (Monari *et al*., 2006). Among the GXM-mediated suppressive effects in macrophages is the upregulation of FasL, which in turn induces apoptosis in T cells expressing Fas (Monari *et al*., 2005). In this study, we analysed the mechanism that causes apoptosis of lymphocytes triggered by FasL/Fas interaction in both *in vitro* and *in vivo* systems.

Results

Role of caspase 8 in apoptosis mediated by GXM-loaded monocytes

To test whether GXM-loaded monocytes (PBM) induced apoptosis via caspase 8 activation, they were incubated with Jurkat cells for 1, 4 and 7 days in presence or absence of caspase 8 inhibitor (Z-IETD-FMK 40 μM). Preliminary experiments showed that the optimal inhibition of caspase 8 was obtained at a dose of 40 μM. Complete inhibition of GXM-induced apoptosis was observed in the presence of caspase 8 inhibitor, Z-IETD-FMK. A complete blockade of apoptosis occurred after 4 days of incubation; this effect was maintained for 7 days of incubation (Fig. 1A–C). Activation of caspase 8 was directly demonstrated by Western blot analysis. Kinetic experiments showed that inactive procaspase 8 was converted into the active form by proteolysis (subunits p43/41 and p18). This activation was evident in GXM-treated cells and was observed at all treatment times (day 1, day 4, day 7) (Fig. 1A–C). The cleavage was partially blocked when caspase 8 inhibitor was used (Fig. 1A–C).

Role of caspase 9 in apoptosis mediated by GXM-loaded monocytes

There are many contexts in which cells die by apoptosis, but the mechanism as to how caspases become activated is still far from clear. Therefore, we analysed the contribution of caspase 9 to the apoptotic process in our experimental system. Apoptosis of Jurkat cells was measured in the presence or absence of a caspase 9 inhibitor (Z-LEHD-FMK 0.1 μM). we observed that inhibition of caspase 9 resulted in a significant decrease in apoptosis in Jurkat cells co-cultured with GXM-loaded monocytes. This inhibition was apparent after 4 and 7 days of incubation, but not after 1 day of incubation (Fig. 2). These results suggest a late and partial involvement of caspase 9 in the observed apoptosis.

GXM-induced caspase 9 activation is dependent on caspase 8 activation

In our experimental system, caspase 8 is induced prior to caspase 9, and a previous report found that caspase 8 can directly activate several caspases, including caspase 9 (Srinivasula *et al*., 1996). As a consequence, we hypothesized that caspase 9 could be activated by caspase 8. Therefore, the inhibitor of caspase 8 was used in our experimental system, and caspase 9 activation was determined. The results (Fig. 3A) show that the activation of caspase 9 was blocked in the presence of an inhibitor of caspase 8.

Given the interdependence between caspase 8 and caspase 9 activation, and given that caspase 8 may induce cleavage of BID – a proapoptotic Bcl-2 member (Creagh *et al*., 2003) and promote the release of mitochondrial cytochrome *c* with subsequent activation of caspase 9 (Li *et al*., 1998), we analysed whether GXM-loaded PBM could activate BID. To this end, untreated and GXM-treated PBM were incubated with Jurkat cells in the absence or presence of caspase 8 inhibitor for 1 day, and Western blotting analysis was performed. The results (Fig. 3B) showed an upregulation of BID activation when Jurkat cells were co-cultured with both GXM-treated or untreated PBM. However, BID activation was more pronounced in GXM-treated PBM than in untreated PBM. In addition, the presence of caspase 8 inhibitor reduced BID activation in GXM–treated PBM but not in GXM untreated PBM, suggesting that PBM induce BID by themselves via other mechanisms.

Caspase 3 is activated by both caspases 8 and 9 in an independent manner

Effector caspases organize the apoptotic process morphologically and biochemically (Stennicke *et al*., 2002); there-

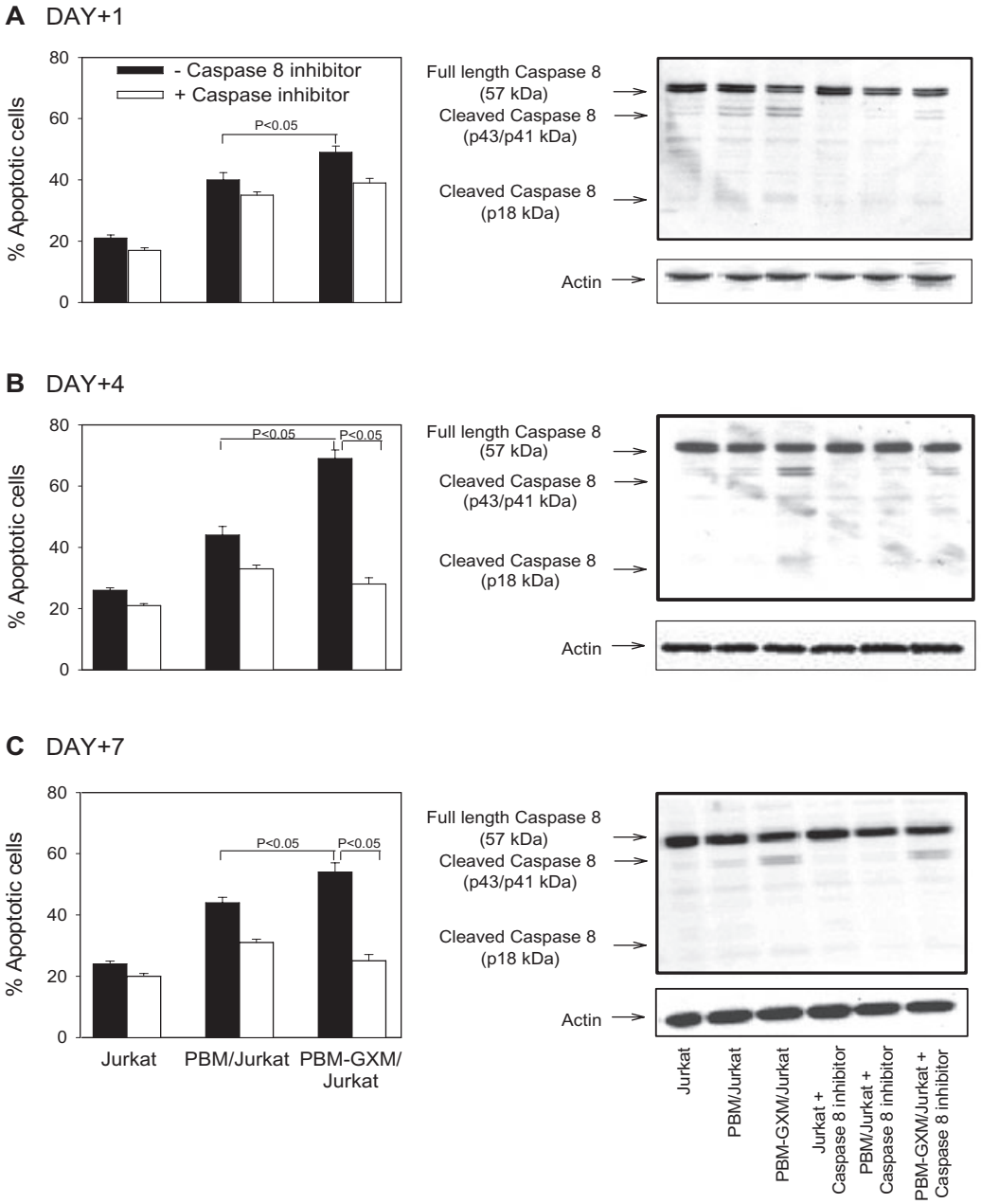

Fig. 1. GXM-loaded PBM induce caspase 8 activation in Jurkat cells. PBM (10^6 cells ml^{-1}) or GXM-PBM (10^6 cells ml^{-1}) were incubated with Jurkat cells (E/T = 1/1) in presence or absence of caspase 8 inhibitor (Z-IETD-FMK, 40 μM) for 1 day (A), 4 days (B) and 7 days (C). At each time point the percentage of apoptotic cells was evaluated by staining with PI, and Western blotting analysis was performed for caspase 8. Cells treated with DMSO were also run in parallel and the results were similar to those obtained in absence of caspase 8 inhibitor. The membrane was re-probed with anti-actin Ab to ensure equal loading in all lines. Bars represent the mean ± SEM of 14 independent experiments. The membranes are representative of five independent experiments with similar results. $P < 0.05$ (caspase 8 inhibitor treated versus caspase 8 inhibitor untreated cells); $P < 0.05$ (PBM-GXM/Jurkat versus PBM/Jurkat).

fore, we analysed whether the effector caspases were activated, and if activation occurred through caspases 8 or 9, or both. The results (Fig. 4) showed that there was an activation of caspase 3 in Jurkat cells exposed to GXM-loaded PBM. This activation was completely blocked by the caspase 8 inhibitor. In addition, an inhibition of caspase 3 was observed by using the caspase 9 inhibitor. This result suggests that caspase 3 is activated, and that caspase 8 is the major caspase orchestrating the cascade that propagates and amplifies the death signal.

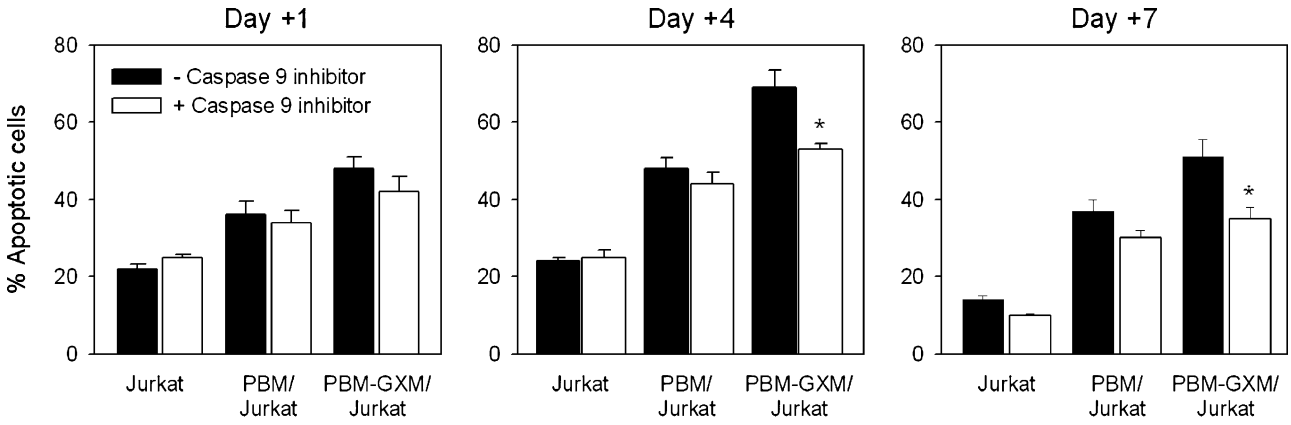

Fig. 2. GXM-loaded PBM induce caspase 9 activation in Jurkat cells. PBM (10^6 cells ml^{-1}) or GXM-PBM (10^6 cells ml^{-1}) were incubated with Jurkat cells (E/T = 1/1) in presence or absence of caspase 9 inhibitor (Z-LEHD-FMK, 0,1 μM) for 1, 4 and 7 days and the percentage of apoptotic cells was evaluated. Cells treated with DMSO were also run in parallel and the results were similar to those obtained in absence of caspase 8 inhibitor. Bars represent the mean ± SEM of seven experiments. $*P < 0.05$ (caspase 9 inhibitor treated versus caspase 9 inhibitor untreated cells).

GXM induces FasL expression and apoptosis in vivo

It has been previously reported that Fas–FasL interactions modulate host defence against systemic *Candida albicans* infection (Netea *et al.*, 1999). Moreover, the absence of Fas–FasL interactions leads to increased proinflammatory cytokine production, resulting in protection against disseminated candidiasis. To verify whether GXM-mediated apoptosis is observed in an *in vivo* experimental system, mice were infected with a virulent *C. albicans* strain (CA-6), and GXM was administered intraperitoneally 5 and 7 days post infection. The day after the last treatment, spleens from five mice were removed, and macrophages and PBL were analysed. Macrophages were analysed for FasL expression, and apoptosis was evaluated in PBL. The results (Fig. 5A and B) showed that FasL expression was upregulated in GXM-treated macrophages from both uninfected and infected mice. In addition, analysis of PBL apoptosis showed that the percentage of apoptotic cells was significantly enhanced in PBL from GXM-treated and *C. albicans* (CA-6)-infected mice compared with PBL from GXM-untreated infected mice (Fig. 5C). Induction of GXM-mediated apoptosis in PBL from *C. albicans*-infected mice was confirmed by the observed activation of caspase 8 in splenic PBL (Fig. 5D).

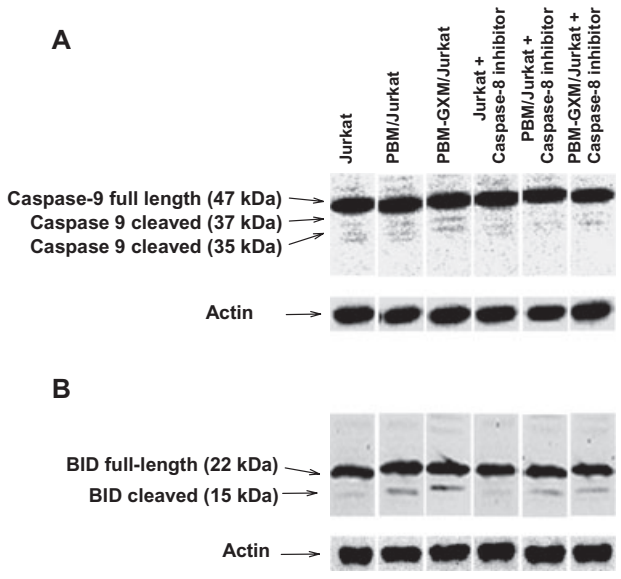

Fig. 3. GXM induces cross-talk between death receptor and mitochondrial pathway. PBM (10^6 cells ml^{-1}) or GXM-PBM (10^6 cells ml^{-1}) were incubated with Jurkat cells (E/T = 1/1) in presence or absence of caspase 8 inhibitor (Z-IETD-FMK, 40 μM) and Western blotting analysis was performed for caspase 9 after 4 days of incubation (A) and for BID after 1 day of incubation (B). Cells treated with DMSO were also run in parallel and the results were similar to those obtained in absence of caspase 8 inhibitor. The membrane was re-probed with anti-actin Ab to ensure equal loading in all lines. The membranes are representative of five independent experiments with similar results.

An additional experiment was carried out to assess the effect of GXM treatment *in vivo* on the production of proinflammatory cytokines in the course of an infection by *C. albicans*. Mice were infected with *C. albicans* and treated with GXM as described above. Spleens were harvested 1, 5 and 7 days after GXM treatment. The tissues were homogenized, and TNF-α and IL-6 levels were tested in supernatant fluids (Fig. 6A and B). The results show a significant decrease in the levels of both cytokines on days 5 and 7 after GXM treatment. Organ culture of kidneys from infected mice was also done in an effort to assess the impact of GXM treatment on the course of *C. albicans* infection. The results show that treatment with GXM resulted in a more severe infection, as demonstrated by a significant (sixfold) increase of *C. albicans* growth in kidneys of GXM-treated mice compared with mice that were not treated with GXM (Fig. 6C).

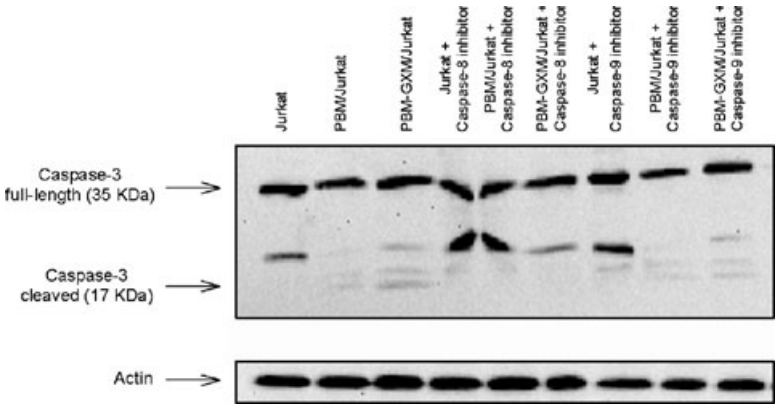

Fig. 4. GXM-loaded PBM induce caspase 3 activation in Jurkat cells. PBM (10^6 cells ml^{-1}) or GXM-PBM (10^6 cells ml^{-1}) were incubated with Jurkat cells (E/T = 1/1) in presence or absence of caspase 8 inhibitor (Z-IETD-FMK, 40 μM) or caspase 9 inhibitor (Z-LEHD-FMK, 0,1 μM) for 4 days. Western blotting analysis for caspase 3 was performed and the membrane was re-probed with anti-actin Ab to ensure equal loading in all lines. Cells treated with DMSO were also run in parallel and the results were similar to those obtained in absence of caspase 8 inhibitor. The membrane is representative of five independent experiments with similar results.

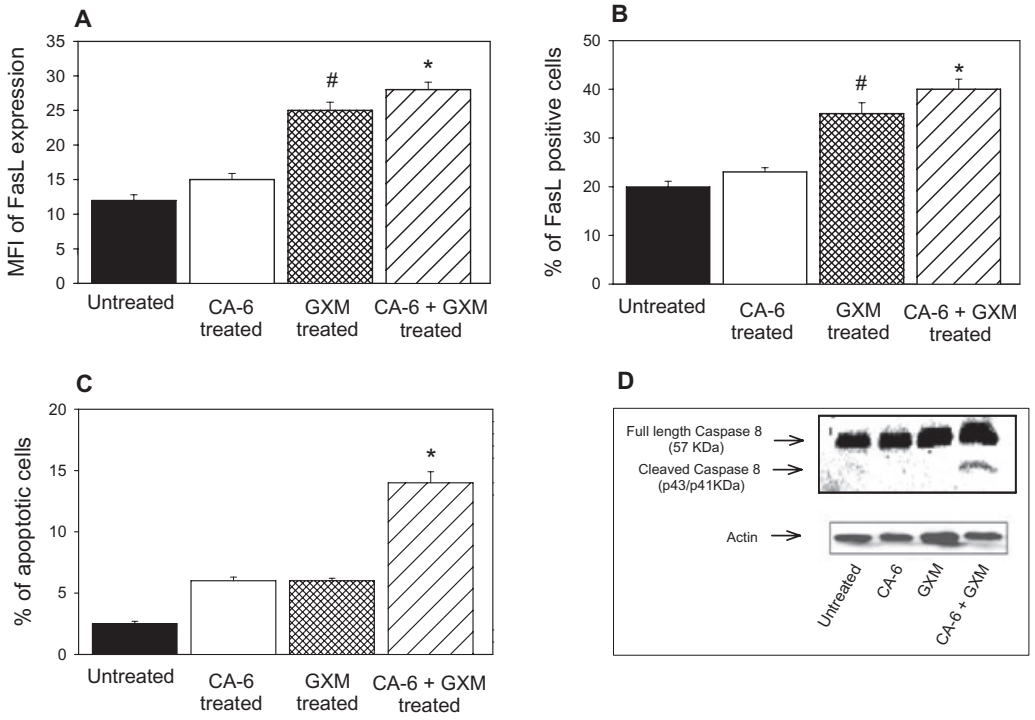

Fig. 5. GXM induces FasL expression in macrophages and apoptosis in PBL from mice.
A and B. Adherent cells from spleen (10^6/tube) of five mice were incubated with rabbit polyclonal antibody to FasL, then with goat Cy3-conjugated anti-rabbit IgG and with FITC-conjugated rat monoclonal antibody anti-mouse CD11b. After incubation, the cells were washed in FB and re-suspended, and macrophages were analysed by flow cytometry to evaluate the mean of fluorescence (A) and the percentage of FasL positive cells (B). Bars represent the mean ± SEM of three experiments. #$P < 0.05$ (mice GXM-treated versus untreated mice); *$P < 0.05$ (mice CA-6 plus GXM-treated versus mice CA-6-treated).
C. PBL recovered from spleen were analysed for apoptotic nuclei as described in *Experimental procedures*. Bars represent the mean ± SEM of three independent experiments. *$P < 0.05$ (mice CA-6 plus GXM treated versus mice CA-6 treated).
D. PBL recovered from spleen, were analysed for caspase 8 activation by Western blotting analysis as described in *Experimental procedures*. The membrane was re-probed with anti-actin Ab to ensure equal loading in all lines. The membrane is representative of three independent experiments with similar results.

Discussion

In a previous report, we demonstrated that GXM, a microbial capsular polysaccharide, induces FasL expression on macrophages, thereby inducing T-cell apoptosis (Monari *et al.*, 2005). In this study, we investigated the activation pathway that regulates the death decision, and whether the apoptotic effect could be translated to an *in vivo* experimental system. Many of the morphological changes in apoptosis are caused by a family of cysteine proteases called caspases which become activated during the apoptotic process (Creagh *et al.*, 2003). In our experimental

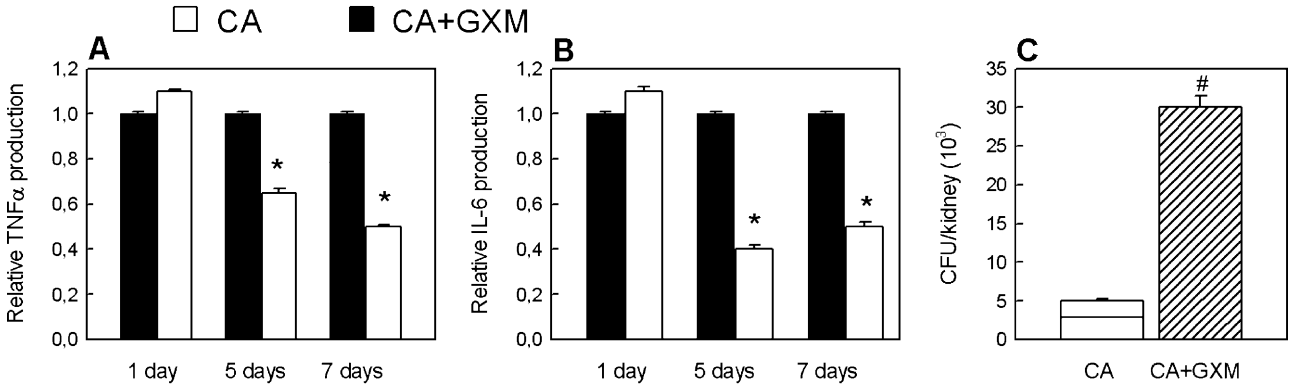

Fig. 6. GXM treatment of *C. albicans* infection induces downregulation of proinflammatory cytokines and produces a more severe infection. Spleens were recovered from mice infected with *C. albicans* and untreated or treated with GXM 1, 5 and 7 days after GXM treatment. The tissues were homogenized, and the supernatant fluids were analysed by ELISA for the production of TNF-α (A) and of IL-6 (B). The graphs represent the mean ± SEM of values obtained from three independent experiments. $*P < 0.05$ (mice CA-6 plus GXM treated versus mice CA-6 treated). (C) Kidneys were recovered from mice infected with *C. albicans* that were untreated or treated with GXM 5 days after GXM treatment, homogenized, diluted and plated in triplicate on Sabouraud agar. The graphs represent the mean ± SEM of values obtained from three independent experiments. $\#P < 0.01$ (mice CA-6 plus GXM treated versus mice CA-6 treated).

system, GXM produces apoptosis via upregulation of FasL, which transduces death signals in Fas-expressing cells. Upon engagement of this receptor, caspase 8 is activated shortly after GXM treatment. Indeed, the activation of caspase 8 was prompt, long lasting and still evident after 7 days of incubation. This is consistent with our previous observations showing a persistent increase in GXM-induced FasL in macrophages (Monari *et al.*, 2005). Therefore, the prolonged and increased apoptosis, as well as the caspase activation could be consequences of long-lasting overexpression of FasL in macrophages. However, we cannot exclude that other receptors, such as TNFR type I and TRAIL-R1, could play a role in caspase 8 activation.

The kinetics of caspase 8 activation were comparable to those observed for apoptosis. The crucial involvement of caspase 8 in GXM-induced apoptosis was demonstrated by the use of a caspase 8 inhibitor that completely abrogated apoptosis. Death receptor-induced activation of caspase 8 may proceed through the involvement of the mitochondrial pathway by activating the Bcl-2 pro-apoptotic protein BID (Li *et al.*, 1997; Luo *et al.*, 1998). Caspase 8 inhibition attenuates receptor-mediated BID activation, demonstrating that caspase 8 directly regulates proteolysis of BID during GXM-induced apoptosis. This is consistent with the observation that caspase 9 activation is dependent on caspase 8 cleavage, indicating that caspase 8 is an essential non-redundant component of the GXM-induced death receptor pathway. GXM-induced apoptosis is therefore mediated by primary activation of caspase 8, which develops a sort of cross-talk with the mitochondrial pathway, leading to caspase 9 activation by release of cytochrome *c*.

Upstream, or apical caspases, including caspases 8 and 9, propagate death signals by activating downstream effector caspases such as caspases 3, 6 and 7 (Slee *et al.*, 1999). GXM-induced apoptosis occurs via downstream effector caspase 3 that is activated by both caspase 8 and caspase 9 in an independent manner. Indeed, the caspase 8 inhibitor completely blocks caspase 3 activation. This is consistent with the fact that caspase 8 is an upstream caspase mainly responsible for initiating caspase activation. However, caspase 9 is also able '*per se*' to orchestrate the activation of caspase 3, contributing to death signal propagation by activating downstream caspases, independently of caspase 8. The importance of caspase 9 activation in GXM-induced apoptosis is substantiated by the observation that the caspase 9 inhibitor significantly reduces apoptosis. Nonetheless, the caspase 8 and caspase 9 pathways cooperate for efficient cell death, in an amplification loop, and they are both activated in the same cell.

The observation that GXM induces apoptosis *in vivo*, as noted in an experimental system in which mice were infected with *C. albicans* and subsequently treated with GXM, suggests that this microbial polysaccharide also functions *in vivo* during infection. This conclusion is supported by caspase 8 activation in lymphocytes of mice treated with GXM and infected with *C. albicans*. Therefore, notwithstanding the complexity of an *in vivo* system, activation of caspase 8 remains a critical step in induction of the GXM-mediated death effect.

The suggested mechanism for GXM-induced apoptosis is summarized in Fig. 7. In particular, apoptosis starts 1 day after GXM treatment. At this time, the death receptor pathway triggers a weak caspase 8 activation, which propagates the death signals through cleavage of effector caspases such as caspase 3. This activation is likely amplified by long-lasting and efficient activation of caspase 8. This results in cross-talk between the extrinsic

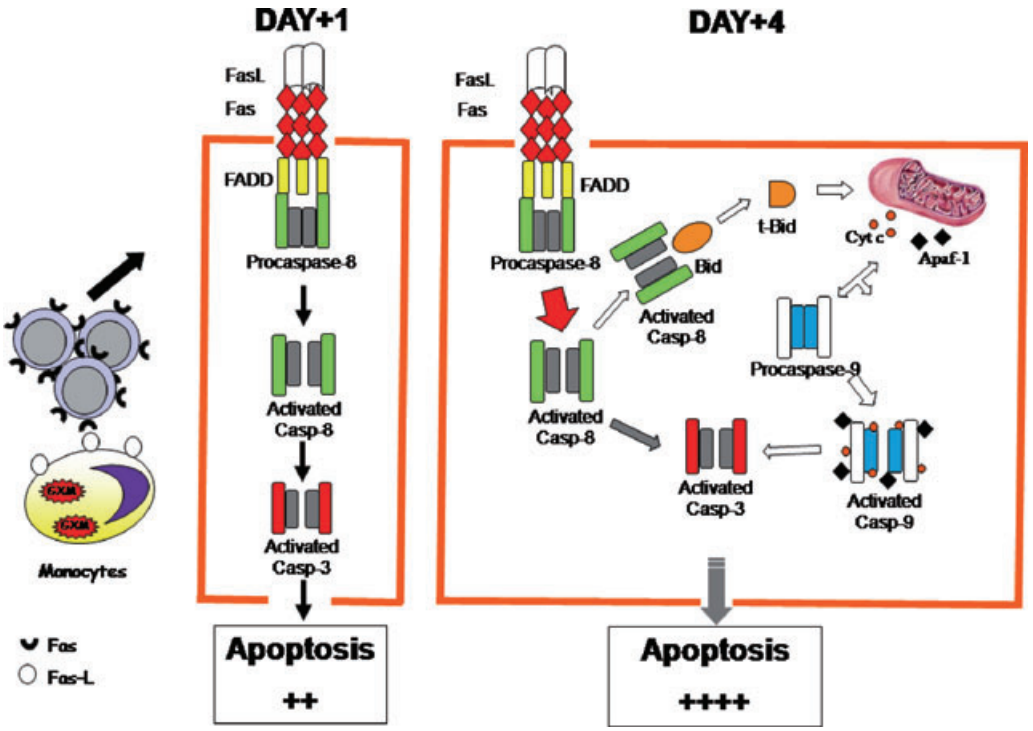

Fig. 7. Kinetics of caspases activation induced by GXM. One day after GXM treatment, the death receptor pathway triggers a weak caspase 8 activation that propagates the death signals through cleavage effector caspases such as caspase 3. This activation is likely amplified by the long-lasting and efficient activation of caspase 8. This results in cross-talk between extrinsic and intrinsic pathways with consequent activation of caspase 9, leading to a strong increase of apoptosis 4 days after GXM treatment. Subsequently, it is possible that feedback mechanisms play a role in the reducing apoptosis that resulted decreased 1 week after GXM treatment.

and intrinsic pathways, with consequent activation of caspase 9 leading to increased GXM-mediated apoptosis that reaches a maximum 4 days post treatment. Subsequently, it is possible that unknown feedback mechanisms play a role in reducing apoptosis which, even though decreased, is still present 1 week after GXM treatment.

The results in this study may explain the rapid reduction in T cells observed in AIDS patients with cryptococcosis (Good and Coax, 1990). Moreover, we observed that GXM induces apoptosis of lymphocytes as well as reduction of proinflammatory cytokine levels, including IL-6 and TNF-α, that mirrors a dramatic increase in microbial growth in target organs (Fig. 6). Taken together, our *in vitro* and *in vivo* studies suggest the potential for this polysaccharide for eliciting tolerance.

Indeed, given (i) that Fas-mediated apoptosis is a major physiologic mechanism which eliminates activated T cells after antigen-stimulated clonal expansion and (ii) that the enhancement of FasL expression facilitates short-term engraftment in a murine model of allogeneic bone marrow (Whartenby *et al*., 2002), GXM could be considered as a potential therapeutic compound in such a clinical setting. In summary, our results identify the mechanism for GXM-induced apoptosis, and indicate that GXM induces FasL induction and apoptosis *in vivo*. These results raise the possibility for a therapeutic role for this microbial polysaccharide in controlling T-cell responses.

Experimental procedures

Reagents

RPMI 1640 with L-glutamine was obtained from Gibco BRL. FCS and penicillin-streptomycin solution were obtained from Sigma. Anti-human caspase 9, caspase 3 and BID rabbit polyclonal Abs, anti-mouse caspase 8 rabbit polyclonal Ab, anti-human caspase 8 mouse mAb, HRP-linked goat anti-rabbit IgG and rabbit anti-mouse IgG were purchased from Cell Signaling Technology. Anti-actin rabbit polyclonal Ab was acquired from Santa Cruz Biotechnology. FITC-conjugated anti-mouse CD11b rat mAb was provided by ImmunoTools. Caspase 8 inhibitor Z-IETD-FMK was purchased from Sigma, and caspase 9 inhibitor Z-LEHD-FMK was purchased from BioVision Research Products. All media used for cell cultures were negative for endotoxins as detected by *Limulus amebocyte* lysate assay (Sigma).

Mice

Eight-week-old female outbred CD1 mice were obtained from Charles River Breeding Laboratories. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of the University of Perugia. They were group housed under

controlled temperature and photoperiod conditions (12 h:12 h, light : dark cycle), and they were allowed unrestricted access to standard mouse chow and tap water. Protocols were approved by the Animal Study Committees of the University of Perugia according to governmental guidelines for animal care.

Infection and GXM treatment

The origin and characteristics of the virulent CA-6 strain have been described previously (Romani *et al*., 1993). After a 24 h culture, yeast cells were diluted to 2.5×10^5 ml^{-1} for intravenous injection via the lateral tail vein in a volume of 0.5 ml mouse^{-1}. Mice were randomly divided into four groups (5 mice/group): untreated, infected with CA-6, treated with GXM and infected with CA-6 plus treated with GXM. A total of two i.p. injections of GXM saline solution (100 μg mouse^{-1}, in 200 μl) was administered; the GXM treatment started 5 days after CA-6 inoculation, and the second inoculation was administered 48 h after the first. Twenty-four hours after the end of treatment, the mice were killed, and the spleens were recovered to evaluate FasL expression, apoptosis and caspase 8 activation.

Cryptococcal polysaccharide

Glucuronoxylomannan was isolated from the culture supernatant fluid of serotype A strain CN 6, as previously described (Houpt *et al*., 1994; Cherniak *et al*., 1980). Isolated soluble GXM contained < 125 pg LPS mg^{-1} of GXM as detected by *Limulus amebocyte* lysate assay (Sigma).

Preparation of PBM

Heparinized venous blood was obtained from healthy donors and diluted with RPMI 1640 plus 10% FCS (cRPMI). Monocytes were recovered as previously described (Monari *et al*., 2003).

Jurkat cells

The human Jurkat T-cell leukaemia cell line was obtained from the ATCC (American Type Culture Collection). Cell lines were maintained as previously described (Monari *et al*., 2005).

Apoptosis

Monocytes (10^6 ml^{-1}) were incubated or not for 48 h with GXM (100 μg ml^{-1}) at 37°C with 5% CO_2 in cRPMI. After incubation, Jurkat cells were added to PBM at an E/T = 1/1. The percentage of Jurkat cells undergoing apoptosis was quantified after 4 days of incubation by staining with propidium iodide (PI). In brief, cells were centrifuged, resuspended in 1.5 ml of hypotonic PI solution (PI, 50 μg ml^{-1} in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma), and kept 1 h at room temperature. The PI-fluorescence of individual nuclei was measured by flow cytometry and the percentage of apoptotic cell nuclei was calculated using FACScan research software (Becton Dickinson, San Jose, CA) (Migliorati *et al*., 1993). To evaluate the role of caspases 8 and 9 on GXM-induced apoptosis, PBM, pre-incubated or not for 48 h with GXM, were incubated with Jurkat cells (E/T = 1/1) in the presence or absence of caspase 8 inhibitor (40 μM) or caspase 9 inhibitor (0,1 μM) for 1, 4 and 7 days in cRPMI at 37°C with 5% of CO_2. After incubation, Jurkat cells apoptosis was evaluated as previously described (Migliorati *et al*., 1993).

Caspases 8, 9 and 3 determination

Jurkat cells were added to PBM, pre-incubated or not for 48 h with GXM in cRPMI at various ratios (E/T = 1/1). After co-culture, the Jurkat cells were recovered, washed, treated with M-PER in the presence of protease inhibitors (Pierce); then lysates were collected. Protein concentrations were determined with a BCA Protein Assay Reagent Kit (Pierce). Lysate proteins at identical concentrations were separated by sodium dodecyl-sulphate-10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Pierce) for Western blot analysis. Membranes were then placed in blocking buffer, incubated overnight at 4°C with either mouse mAb to caspase 8 (1:1000), or with rabbit polyclonal Ab to caspase 9 (1:1000), or with rabbit polyclonal Ab to caspase 8 (1:1000) or with rabbit polyclonal Ab to caspase 3 (1:1000) in blocking buffer. Detection was then achieved with the appropriate secondary Abs coupled to HRP, followed by Chemi-Lucent Trial Kit (Chemicon International). Immunoblotting with the rabbit polyclonal anti-actin was used as an internal loading control to ensure equivalent amounts of protein in each lane. Immunoreactive bands were visualized and quantified by Chemi-doc Instrument (Bio-Rad).

FasL expression, apoptosis and caspase 8 determination in vivo

FasL expression was evaluated on splenic macrophages ($CD11b^+$ cells). Twenty-four hours after the end of GXM treatment, the mice were killed and the spleens were recovered, homogenized, filtered by Cell Strainer (BD, Biosciences, USA) and centrifuged. The cell pellet was treated with hypotonic saline buffer to lyse the erythrocytes. FasL expression on macrophages was determined as described (Monari *et al*., 2005).

To evaluate PBL apoptosis in cells from spleens of infected or uninfected mice, the mice were killed and spleens were removed 24 h after the end of GXM treatment. Spleens were homogenized, filtered and centrifuged. The cell pellet was treated with hypotonic saline buffer to lyse the erythrocytes, washed and plated in six-well plates (Falcon, Becton Dickinson) at a density of 2×10^6 ml^{-1} in cRPMI. After a 2 h incubation, PBL were recovered, and were evaluated for apoptosis as described (Migliorati *et al*., 1993) and caspase 8 activation as described above.

TNF-α and IL-6 determination

Spleens recovered 1, 5 and 7 days after GXM treatment from mice infected with *C. albicans* were homogenized and centrifuged. Supernatant fluids were sterilized by passage through a Millipore filter and stored at –20°C. TNF-α and IL-6 concentrations were determined by the use of commercial enzyme-linked immunoassay kits from BioSource according to the manufacturer's recommendations.

C. albicans *growth in kidneys*

Candida albicans growth in kidneys of infected mice, both untreated and treated with GXM, was determined 5 days after

GXM treatment. Kidneys were removed and homogenized. The samples were diluted, plated in triplicate on Sabouraud agar (BioChemika Fluka), and the results were expressed as numbers of CFU per organ.

Statistical analysis

Data are reported as the mean ± SEM from replicate experiments. Data were evaluated by one-way analysis of variance (ANOVA). *Post hoc* comparisons were carried out with Bonferroni's test.

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References

Askenasy, N., Yolcu, E.S., Yaniv, I., and Shirwan, H. (2005) Induction of tolerance using Fas ligand: a double-edged immunomodulator. *Blood* **105:** 1396–1404.

Cherniak, R., Reiss, E., Slodki, M.E., Plattner, R.D., and Blumer, S.O. (1980) Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. *Mol Immunol* **17:** 1025–1032.

Creagh, E.M., Conroy, H., and Martin, S.J. (2003) Caspase-activation pathways in apoptosis and immunity. *Immunol Rev* **193:** 10–21.

Good, C.B., and Coax, W.A. (1990) Cryptococcal infections in patients with AIDS. *N Engl J Med* **322:** 701–702.

Green, D.R. (2003) Overview: apoptotic signaling pathways in the immune system. *Immunol Rev* **193:** 5–9.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R., and Ferguson, T.A. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270:** 1189–1192.

Houpt, D.C., Pfrommer, G.S., Young, B.J., Larson, T.A., and Kozel, T.R. (1994) Occurrences, immunoglobulin classes, and biological activities of antibodies in normal human serum that are reactive with *Cryptococcus neoformans* glucuronoxylomannan. *Infect Immun* **62:** 2857–2864.

Kavurma, M.M., and Khachigian, L.M. (2003) Signaling and transcriptional control of Fas ligand gene expression. *Cell Death Differ* **10:** 36–44.

Krammer, P.H. (2000) CD95's deadly mission in the immune system. *Nature* **407:** 789–795.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91:** 479–489.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94:** 491–501.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell* **94:** 481–490.

Migliorati, G., Nicoletti, I., Pagliacci, M.C., D'Adamio, L., and Riccardi, C. (1993) Interleukin-4 protects double-negative and CD4 single-positive thymocytes from dexamethasone-induced apoptosis. *Blood* **81:** 1352–1358.

Monari, C., Kozel, T.R., Paganelli, F., Pericolini, E., Perito, S., Bistoni, F., *et al.* (2006) Microbial immune suppression mediated by direct engagement of inhibitory Fc receptor. *J Immunol* **177:** 6842–6851.

Monari, C., Pericolini, E., Bistoni, G., Casadevall, A., Kozel, T.R., and Vecchiarelli, A. (2005) *Cryptococcus neoformans* capsular glucuronoxylomannan induces expression of fas ligand in macrophages. *J Immunol* **174:** 3461–3468.

Monari, C., Retini, C., Casadevall, A., Netski, D., Bistoni, F., Kozel, T.R., and Vecchiarelli, A. (2003) Differences in outcome of the interaction between *Cryptococcus neoformans* glucuronoxylomannan and human monocytes and neutrophils. *Eur J Immunol* **33:** 1041–1051.

Nagata, S. (1999) Fas ligand-induced apoptosis. *Annu Rev Genet* **33:** 29–55.

Nagata, S., and Suda, T. (1995) Fas and Fas ligand: lpr and gld mutations. *Immunol Today* **16:** 39–43.

Netea, M.G., van Der Meer, J.W., Meis, J.F., and Kullberg, B.J. (1999) Fas–FasL interactions modulate host defense against systemic *Candida albicans* infection. *J Infect Dis* **180:** 1648–1655.

Oyaizu, N., Adachi, Y., Hashimoto, F., McCloskey, T.W., Hosaka, N., Kayagaki, N., *et al.* (1997) Monocytes express Fas ligand upon CD4 cross-linking and induce CD4+ T cells apoptosis: a possible mechanism of bystander cell death in HIV infection. *J Immunol* **158:** 2456–2463.

Romani, L., Mencacci, A., Cenci, E., Spaccapelo, R., Mosci, P., Puccetti, P., and Bistoni, F. (1993) CD4+ subset expression in murine candidiasis. Th responses correlate directly with genetically determined susceptibility or vaccine-induced resistance. *J Immunol* **150:** 925–931.

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., *et al.* (1999) Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* **144:** 281–292.

Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E.S. (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proc Natl Acad Sci USA* **93:** 14486–14491.

Stennicke, H.R., Ryan, C.A., and Salvesen, G.S. (2002) Reprieval from execution: the molecular basis of caspase inhibition. *Trends Biochem Sci* **27:** 94–101.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356:** 314–317.

Whartenby, K.A., Straley, E.E., Kim, H., Racke, F., Tanavde, V., Gorski, K.S., *et al.* (2002) Transduction of donor hematopoietic stem-progenitor cells with Fas ligand enhanced short-term engraftment in a murine model of allogeneic bone marrow transplantation. *Blood* **100:** 3147–3154.

