Fas Ligand, Bcl-2, Granulocyte Colony-Stimulating Factor, and p38 Mitogen-activated Protein Kinase: Regulators of Distinct Cell Death and Survival Pathways in Granulocytes

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Abstract

The short life span of granulocytes, which limits many inflammatory responses, is thought to be influenced by the Bcl-2 protein family, death receptors such as CD95 (Fas/APO-1), stress-activated protein kinases such as p38 mitogen-activated protein kinase (MAPK), and proinflammatory cytokines like granulocyte colony-stimulating factor (G-CSF). To clarify the roles of these various regulators in granulocyte survival, we have investigated the spontaneous apoptosis of granulocytes in culture and that induced by Fas ligand or chemotherapeutic drugs, using cells from normal, CD95-deficient *lpr*, or *vav-bcl-2* transgenic mice. CD95-induced apoptosis, which required receptor aggregation by recombinant Fas ligand or the membrane-bound ligand, was unaffected by G-CSF treatment or Bcl-2 overexpression. Conversely, spontaneous and drug-induced apoptosis occurred normally in *lpr* granulocytes but were suppressed by G-CSF treatment or Bcl-2 overexpression. Although activation of p38 MAPK has been implicated in granulocyte death, their apoptosis actually was markedly accelerated by specific inhibitors of this kinase. These results suggest that G-CSF promotes granulocyte survival largely through the Bcl-2–controlled pathway, whereas CD95 regulates a distinct pathway to apoptosis that is not required for either their spontaneous or drug-induced death. Moreover, p38 MAPK signaling contributes to granulocyte survival rather than their apoptosis.

Key words: apoptosis • Bcl-2 • Fas ligand • p38 mitogen-activated protein kinase • granulocyte colony-stimulating factor

Introduction

Production of granulocytes from common myeloid precursors in the bone marrow is regulated in part by cytokines such as G-CSF and GM-CSF (1). After their release into the blood stream, granulocytes can migrate into tissues where they act as phagocytes of bacteria. The inflammatory responses mediated by granulocytes are curtailed by their short life span, which is determined by an intrinsic suicide program involving activation of proteases of the caspase family. The programmed demise of these cells prevents excessive release of histotoxic substances, and promotes subsequent clearance of the remaining apoptotic bodies by macrophages (2, 3). The biologic mechanisms that regulate granulocyte apoptosis are poorly understood, although it is well known that their survival is promoted by inflammatory cytokines such as G-CSF, GM-CSF, and IL-5, as well as by microbial substances, like LPS (1, 4). The pathways controlling their viability have been variously suggested to involve the Bcl-2 family of intracellular proteins (5–7), death receptors (8–10), and stress-activated protein kinases (11, 12).

The Bcl-2 family of proteins has been shown to regulate granulocyte survival. Although Bcl-2 and Bcl-X_L are expressed at very low or undetectable levels in granulocytes (5, 13, 14), these cells do express the related antiapoptotic family members Mcl-1 and A1 (5–7). Moreover, levels of Mcl-1 and A1 are upregulated by proinflammatory cytokines or LPS, and Mcl-1 levels in myeloid progenitor cells decline upon growth factor withdrawal (5, 15). In addition, granulocytes from mice lacking one of the genes for A1 undergo accelerated apoptosis when cultured in simple medium, but exhibit normal sensitivity to TNF (16).

Granulocytes bear CD95 (Fas/APO-1) and TNF-R1 on their surfaces, and the Ab- or ligand-mediated activation of

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these receptors triggers their apoptotic death (8–10). The observation that antagonistic Abs to CD95 (Fas/APO-1) delayed spontaneous apoptosis of human granulocytes in culture led to the suggestion that the Fas ligand (FasL)/CD95 system modulates their life span (9, 17, 18). This notion, however, has been challenged by reports which demonstrated that spontaneous granulocyte death, granulocyte cellularity, and the colony-forming potential of myeloid precursors are all normal in CD95-deficient lymphoproliferation (*lpr*[1] [19]) and FasL mutant generalized lymphadenopathy (*gld*) mice (20).

The stress-activated kinase p38 mitogen-activated protein kinase (MAPK), a serine/threonine kinase whose targets include the activating transcription factor 2 (ATF-2) and myocyte enhancer factor 2 (MEF-2) transcription factors (21), is activated in dying granulocytes (11, 12). Inhibition of p38 MAPK activity using synthetic inhibitors was reported to delay neutrophil apoptosis in culture, whereas blocking the related extracellular signal–regulated kinase (ERK) pathway had no effect (11, 12). This observation led to the assumption that activation of p38 MAPK is a prerequisite for granulocyte apoptosis.

To explore how these different signaling pathways interact in regulating the life span of granulocytes, we have analyzed the survival of resting and peritoneal exudate granulocytes derived from wild-type (*wt*), CD95-deficient *lpr* mutant, and *vav-bcl-2* transgenic mice, both during culture in simple medium and after treatment with FasL or chemotherapeutic drugs. We have also investigated how signals from G-CSF and p38 MAPK impact granulocyte apoptosis. Our results demonstrate that multimerization of CD95 (Fas/APO-1) is required to induce cell death in granulocytes. We observed that activation of granulocytes with G-CSF or overexpression of Bcl-2 delayed spontaneous and drug-induced apoptosis but not FasL-mediated death, whereas only the latter was prevented by the absence of CD95 (Fas/APO-1). FasL and Bcl-2, therefore, regulate distinct apoptotic pathways in granulocytes. We further demonstrate that signaling through p38 MAPK, rather than promoting apoptosis in granulocytes, as previously reported, is required to maintain their survival.

Materials and Methods

Mice. The generation of the *vav-bcl-2*-68 and -69 transgenic mouse strains expressing a human *bcl-2* cDNA under control of the *vav* promoter at high levels in all hematopoetic cell types has been described (22). All C57BL/6 and C57BL/6 *lpr* mice were bred in The Walter and Eliza Hall Institute Animal Facility in Kew (Victoria) and were used between 6 and 12 wk of age.

Cell Culture and Reagents. Neuro2A neuroblastoma cells stably expressing membrane-bound mouse FasL, and control Neuro2A cells expressing the neomycin resistance gene have been described (23). Resting granulocytes were isolated from the bone marrow, and peritoneal exudate granulocytes were collected by lavage of the peritoneal cavity of mice that had been injected intraperitoneally 3 h earlier with 2 ml of a 0.5% casein/PBS solution. Granulocytes and Neuro2A cells were cultured in the high glucose version of DMEM supplemented with 13 μM folic acid, 250 μM l-asparagine, 50 μM 2-ME, and 10% FCS (TRACE). Human G-CSF (Granocyte™) was provided by AMRAD. The pan-caspase inhibitor zVADfmk, the p38 MAPK inhibitors SB203850 and SB202190, and the MAPK kinase (MEK)-1 inhibitor PD98059 were purchased from Calbiochem. Production of recombinant FLAG-tagged FasL has been described (24). Etoposide, doxorubicin, and cis-platin were obtained from David Bull Laboratories and DellaWest.

Determination of MAPK Activation and Immunoblotting. Cells (10^6) were resuspended in 50 μl lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, pH 7.5, supplemented with 1 mM Na_3VO_4, 1 mM NaF, 25 μg/ml aprotinin, leupeptin, pepstatin, and Pefabloc, and 1% Triton X-100; Sigma-Aldrich). Samples were cleared by centrifugation (16,000 *g*, 5 min, 4°C). Proteins were size-fractionated on SDS-PAGE 4–20% gradient gels under reducing conditions (Novex) and transferred onto polyvinyl-difluoridon membranes (Millipore) by electroblotting. The membranes were incubated for >12 h at 4°C in blocking buffer (PBS containing 0.05% Tween-20 and 5% skim milk). The anti–phospho-p38 MAPK, p38 MAPK, phospho–c-Jun NH_2-terminal kinase (JNK), phospho-ERK, and ERK kinase Abs (New England Biolabs, Inc.), were diluted at 1:1,000 in blocking buffer for immunolabeling. The mAbs to mouse Bcl-2 (BD PharMingen), Bcl-X_L (a gift from C. Thompson, University of Pennsylvania, Philadelphia, PA), Bax (BD PharMingen), Bcl-w (16H12) (25), or Bim (26) were diluted and used at 2–5 μg/ml in blocking buffer. Peroxidase-conjugated rabbit anti–mouse Ig Abs (Silenus) or goat anti–rat Ig Abs (Southern Biotechnology Associates, Inc.) served as secondary reagents (1:1,000), and the enhanced chemiluminescence system was used for detection. To demonstrate equal protein loading of the gel, membranes were reprobed with polyclonal goat antiserum sc-535-G specific for p38 MAPK (Santa Cruz Biotechnology, Inc.), an antiserum specific for ERK1 and ERK2 (New England Biolabs, Inc.), or a mouse mAb to heat shock protein (Hsp)70 (a gift from R. Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia).

Cell Sorting and Immunofluorescence Analysis. Granulocytes were stained with FITC-labeled rat anti–Gr-1 mAb RB6-8C5 (5 μg/ml) in PBS/10% FCS, 30 min on ice) and sorted on a MoFlow sorter (Cytomation) after gating on the basis of the forward and side light scatter and exclusion of dead cells using propidium iodide staining. Sorted granulocytes from the bone marrow and the peritoneal cavity were >98% positive for Gr-1 and Mac-1, but negative for the B cell marker B220, the T cell marker Thy-1, the erythroid cell marker TER-119, and the macrophage marker F4/80.

Expression of transgenic human Bcl-2 protein was assessed in granulocytes sorted by staining bone marrow cells with Abs to Thy1, B220, Ter119, and F4/80 to exclude nongranulocytic cells from analysis, followed by cytoplasmic immunofluorescence staining (27). Cells (0.2×10^6) were fixed for 10 min at room temperature in 1 ml 1% paraformaldehyde in PBS. After washing twice with PBS, cells were resuspended in FACS® buffer (balanced salt solution, 2% FCS, 10 mM sodium azide) containing 0.3% saponin (Sigma-Aldrich) and 2 μg/ml mouse anti–human Bcl-2 mAb, Bcl-2-100 (a gift from D. Mason, University of Ox-

[1]*Abbreviations used in this paper:* ERK, extracellular signal regulating kinase; FADD, Fas-associated death domain; GST, glutathione *S*-transferase; Hsp, heat shock protein; JNK, c-jun NH_2-terminal kinase; *lpr*, lymphoproliferation; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NF, nuclear factor; *wt*, wild-type.

ford, Oxford, UK). After 30 min on ice, cells were washed in FACS® buffer containing 0.03% saponin and incubated with FITC-labeled goat anti–mouse IgG–specific Ab (5 μg/ml in FACS® buffer containing 0.3% saponin) for 30 min on ice. After a final wash, the cells were resuspended in 200 μl FACS® buffer and 5,000 cells per sample were analyzed on a FACScan™ (Becton Dickinson). Cells from C57BL/6 mice stained with Bcl-2-100 mAb served as a negative control for transgene expression.

Expression of mouse Bcl-2, Bcl-X_L, and Bax proteins was assessed in granulocytes sorted by staining bone marrow cells with Abs to Thy1, B220, Ter119, and F4/80 to exclude nongranulocytic cells from analysis, followed by cytoplasmic immunofluorescence staining (27) using the following mAbs: hamster anti–mouse Bcl-2 (BD PharMingen), mouse anti-Bax (BD PharMingen), or mouse anti–Bcl-X_L Abs. FITC-labeled goat anti–mouse IgG Abs or goat anti–hamster IgG Abs (BD PharMingen) were used as secondary reagents at 1:100 dilution in FACS® buffer containing 0.3% saponin. Expression of CD95 (Fas/APO-1) and FasL on naive granulocytes or Neuro2A cells was determined by surface immunofluorescence staining using mAbs specific to mouse CD95 (Fas/APO-1) (JO-2; BD PharMingen) and FasL (Kay-10; BD PharMingen). FITC-labeled goat anti–mouse IgG Abs or goat anti–hamster IgG Abs (Silenus) were used as secondary reagent. Samples stained with secondary reagent alone served as a negative control.

Cell Death Assays. The percentage of viable cells in culture was determined by staining with 2 μg/ml propidium iodide and analyzing the samples on a FACScan™ (Becton Dickinson). Alternatively, cells were stained with trypan blue (0.1% in PBS) and analyzed in a hemocytometer. As cell death–inducing stimuli we used coculture with FasL-expressing Neuro2A cells or control Neuro2A cells, 1–1,000 ng/ml FLAG-tagged recombinant human FasL (24), 10–50 μg/ml etoposide, 50 μg/ml cis-platin, or 500 ng/ml doxorubicin. Recombinant FasL was aggregated by the addition of 0.5 μg/ml M2 anti-FLAG mAb (Sigma-Aldrich).

Statistical Analysis. Degrees of significance for the comparison of effects of FasL, G-CSF, drug treatment, or Bcl-2 overexpression on survival of granulocytes were assessed using the Student's paired *t* test and a Microsoft Excel software program. $P < 0.04$ was considered to indicate statistically significant differences.

Results

Receptor Multimerization Is Needed for FasL-induced Apoptosis. It has been shown that CD95-induced apoptosis in lymphocytes requires receptor multimerization either by the physiological membrane-bound FasL or by aggregated recombinant FasL. In contrast, soluble FasL did not kill cells, and at high concentrations could even act as an antagonist of membrane-bound FasL (23, 24, 28, 29). To assess whether these observations apply only to lymphocytes or are of general importance, we compared the death-inducing potency of soluble or aggregated FasL on granulocytes from bone marrow of *wt* and CD95-deficient mutant *lpr* mice. Immunofluorescence staining and flow cytometric analysis revealed that CD95 is expressed on granulocytes from *wt* mice (Fig. 1 a) but, as expected, not on those from *lpr* mice (30; Fig. 1 b). Only multimerized or membrane-bound FasL could induce significant killing of *wt* granulocytes (Fig. 1 c). This effect was exclusively mediated via CD95, since cells from *lpr* mice were completely resistant

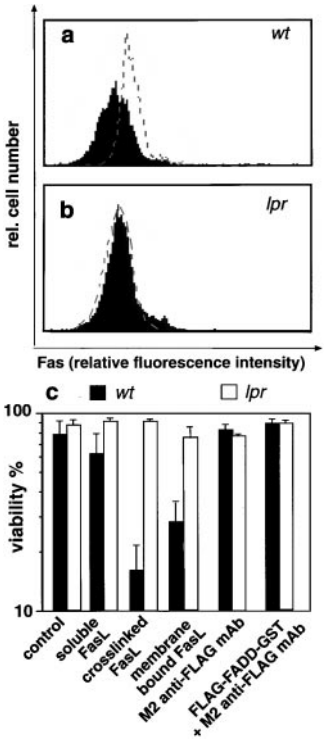

Figure 1. Receptor multimerization is required for FasL-induced killing of granulocytes. CD95 expression was determined on sorted bone marrow–derived granulocytes from (a) *wt* and (b) *lpr* mice by indirect immunofluorescence staining and flow cytometric analysis. Control staining is shown by filled histograms. Specific staining for CD95 is shown by open histograms. (c) Sorted granulocytes from bone marrow of *wt* or *lpr* mice were cultured for 24 h in the absence or presence of 100 ng/ml soluble FasL or multimerized FasL or with Neuro2A cells expressing membrane-bound FasL. Treatment with M2 anti-FLAG mAb (500 ng/ml) with or without a FLAG–FADD-GST fusion protein (1 μg/ml) served as additional controls. Viability was assessed by trypan blue staining and analysis in a hemocytometer. Data shown represent arithmetic means ± SD of three independent experiments and animals. A statistically significant degree of cell killing was only observed in *wt* cells treated with either multimerized ($P < 0.0003$) or membrane-bound FasL ($P < 0.009$).

to such treatment (Fig. 1 c). To exclude a nonspecific toxic effect of the M2 anti-FLAG mAb or the FasL–anti-FLAG mAb immune complex on granulocytes, we showed that neither the M2 anti-FLAG mAb alone nor immune complexes of FLAG–Fas-associated death domain(FADD)–glutathione *S*-transferase (GST) fusion proteins plus M2 anti-FLAG mAb killed granulocytes (Fig. 1 c).

These results demonstrate that FasL-induced apoptosis in granulocytes requires receptor multimerization and not simply trimerization.

CD95 and Bcl-2 Regulate Distinct Pathways to Apoptosis in Granulocytes. It has been controversial whether members of the Bcl-2 family can regulate CD95-induced apoptosis. Although some reports have indicated that CD95-induced apoptosis in lymphocytes or hepatocytes could be regulated by Bcl-2 and related proteins (31–33), we and others have shown that CD95 and the Bcl-2 family regulate two distinct pathways to cell death (27, 34–38). To determine whether Bcl-2 can modulate FasL-induced death in granulocytes, we exploited *vav-bcl-2* transgenic mice, which express a human *bcl-2* cDNA under control of the *vav* promoter in all hematopoietic cell types (22). Expression of the transgene-encoded human Bcl-2 protein in granulocytes was confirmed by immunofluorescence staining and flow cytometric analysis (Fig. 2, a and b). The effect of Bcl-2 on FasL-induced apoptosis was analyzed by comparing survival of granulocytes from *wt*, CD95-deficient *lpr*, and *vav-bcl-2* transgenic mice. Multimerized FasL killed *wt* granulocytes in a dose- and time-dependent manner (Fig. 2, c and h).

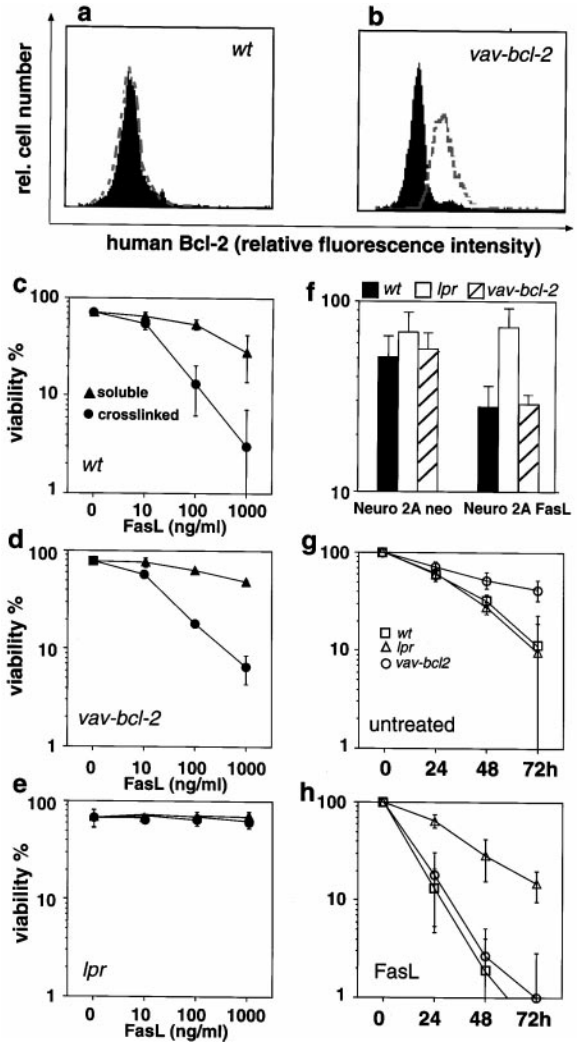

Figure 2. Bcl-2 and FasL regulate distinct pathways to apoptosis in granulocytes. Expression of transgene-encoded human Bcl-2 was determined on sorted bone marrow–derived granulocytes from (a) *wt* and (b) *vav-bcl-2* transgenic mice by indirect immunofluorescence staining and flow cytometric analysis. The Bcl-2-100 Ab used recognizes specifically human but not mouse Bcl-2. Control staining is shown by filled histograms and specific staining for human Bcl-2 by open histograms. Granulocytes from bone marrow of (c) *wt*, (d) *vav-bcl-2* transgenic, or (e) *lpr* mice were sorted and treated with graded concentrations of soluble FasL or FasL multimerized with M2 anti-FLAG mAb. Alternatively, granulocytes were cocultured with (f) control (neo) or FasL-expressing Neuro2A cells. Cell viability was determined after 24 h. In a kinetic analysis, sorted granulocytes were cultured in the (g) absence or (h) presence of 100 ng/ml multimerized FasL for 24, 48, or 72 h. Cell viability was assessed by propidium iodide staining and flow cytometric analysis. Data shown represent arithmetic means ± SD of four independent experiments and animals. There are no statistically significant differences between FasL-treated *wt* and *bcl-2* transgenic cells regardless of time of treatment or the FasL concentration applied. Survival of granulocytes from *vav-bcl-2* transgenic mice in simple culture medium was significantly better compared with that of granulocytes from *wt* or *lpr* mice ($P < 0.006$).

Notably, no difference was observed between *wt* and *bcl-2* transgenic cells (Fig. 2, c, d, and h) but, as expected, CD95-deficient *lpr* cells were completely resistant to FasL (Fig. 2, e and h). The cytotoxicity observed after treatment of granulocytes from *wt* and *vav-bcl*-2 transgenic mice with high concentrations of soluble FasL indicates that some aggregation of CD95 occurs in the absence of cross-linking Ab.

It is conceivable that recombinant FasL multimerized by an anti-FLAG Ab does not perfectly mimic the physiological membrane-bound FasL. Therefore, we cocultured granulocytes with the CD95-negative neuroblastoma cell line Neuro2A, engineered to express membrane-bound FasL (24). Like the multimerized recombinant FasL, membrane-bound FasL induced apoptosis in granulocytes from *wt* and *vav-bcl-2* but not in those from *lpr* mice (Fig. 2 f). Coculture with control Neuro2A cells transfected with a *neo* construct did not significantly affect granulocyte survival (Fig. 2 f). A time course experiment confirmed the equal sensitivity of *wt* and *bcl-2* transgenic granulocytes to FasL-induced killing, whereas *lpr* granulocytes were resistant to this treatment (Fig. 2, e and h)

To prove that the transgenic Bcl-2 protein was functional, we cultured granulocytes from *vav-bcl-2* mice in the absence of supportive cytokines, or treated them with the chemotherapeutic anticancer drugs etoposide, cis-platin, or doxorubicin. In contrast to FasL-induced apoptosis, the spontaneous cell death, as well as drug-induced death, was significantly inhibited by overexpression of Bcl-2 (Fig. 2 g and Fig. 3, a and b).

The FasL/CD95 system has been implicated in the spontaneous death of granulocytes (9) and in drug-induced cell killing (39). Therefore, we assessed the contribution of the FasL/CD95 system to spontaneous and drug-induced killing of granulocytes by comparing survival of cells from *wt* and CD95-deficient *lpr* mice. The granulocytes were either treated with etoposide, cis-platin, or doxorubicin for up to 72 h or were left untreated. We detected no differences in spontaneous death rates or drug-induced killing between granulocytes from *wt* and *lpr* mice (Fig. 2 g and Fig. 3, a and c).

We also investigated whether the tissue localization (and perhaps activation status) influences the sensitivity of granulocytes to spontaneous death in culture or to treatment with FasL or antitumor drugs. Therefore, mice were injected intraperitoneally with casein and granulocytes were sorted from peritoneal exudate cells. We observed that spontaneous death of peritoneal exudate granulocytes in culture was accelerated compared with resting granulocytes from the bone marrow (Fig. 3, a and d). Peritoneal exudate granulocytes were sensitive to FasL, and Bcl-2 provided no protection (Fig. 3, d and e), although it significantly delayed spontaneous and etoposide-induced apoptosis in these cells (Fig. 3, d and e). On the other hand, the spontaneous and drug-induced death of peritoneal exudate *lpr* granulocytes was not affected by the absence of CD95 (Fig. 3 f).

Collectively, these results demonstrate that CD95 and Bcl-2 regulate distinct pathways to apoptosis in granulocytes, and that the FasL/CD95 system is not required for their spontaneous or drug-induced death. Conversely, Bcl-2 is a potent inhibitor of these pathways to cell death but has no impact on FasL-induced killing.

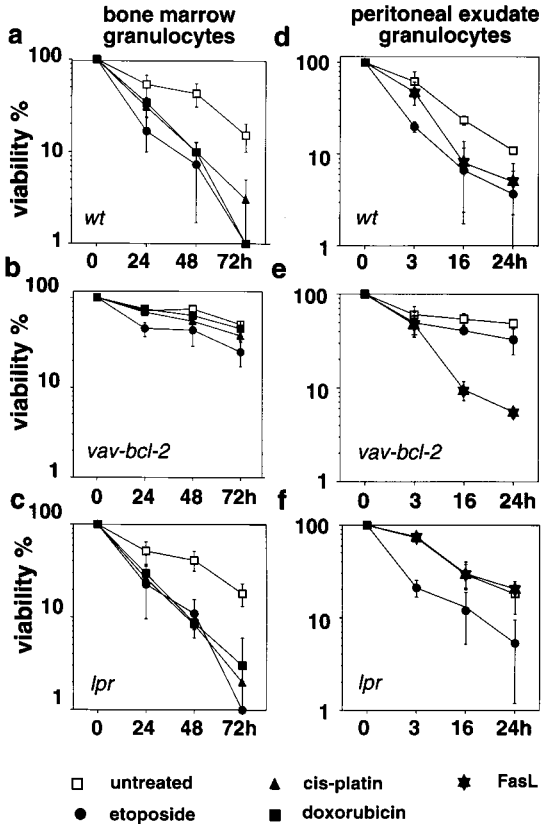

Figure 3. Bcl-2 inhibits spontaneous and drug-induced apoptosis in bone marrow and peritoneal exudate granulocytes. (a–c) Granulocytes from bone marrow or (d–f) peritoneal exudate of casein-injected (a and d) *wt*, (b and e) *vav-bcl-2* transgenic, or (c and f) *lpr* mice were sorted and cultured with the anticancer drugs etoposide (10 μg/ml), cis-platin (50 μg/ml), doxorubicin (0.5 μg/ml), multimerized FasL (100 ng/ml), or were left untreated. Cell viability was determined at the indicated times as described in the legend to Fig. 2. Data shown represent arithmetic means ± SD of four independent experiments and four to seven animals. There was no statistically significant difference between FasL-treated *wt* or *bcl-2* transgenic granulocytes, regardless of time point analyzed and origin of the cells ($P > 0.05$ or higher). Granulocytes from *vav-bcl-2* transgenic mice died at significantly slower rates when cultured in simple medium or after drug treatment than granulocytes derived from *wt* or *lpr* mice, again regardless of their activation status ($P < 0.03$ or lower). No significant difference was observed between *wt*- and *lpr*-derived granulocytes after drug treatment ($P > 0.13$ or higher).

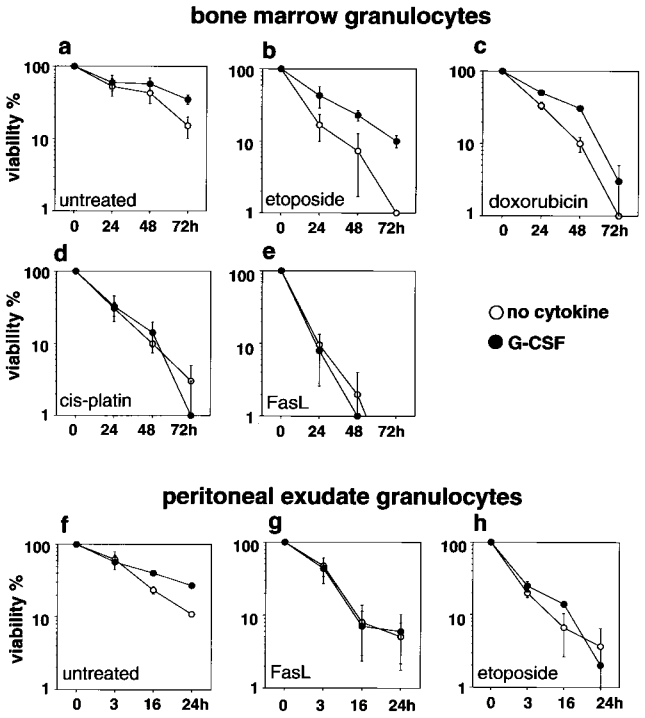

Figure 4. G-CSF inhibits spontaneous and drug-induced apoptosis of granulocytes. (a–e) Granulocytes were isolated from bone marrow (f–h) or peritoneal cavity of *wt* mice that had been injected with casein. Cells were cultured in the absence (open circles) or presence of 100 ng/ml human G-CSF (filled circles), which was added 30 min before the addition of death stimuli. Cells were cultured in (a and f) medium alone or (b and h) in the presence of 10 μg/ml etoposide, (c) 50 μg/ml cis-platin, (d) 0.5 μg/ml doxorubicin, or (e and g) 100 ng/ml cross-linked FasL. Cell viability was assessed as in the legend to Fig. 2. Data shown represent arithmetic means ± SD of three to four independent experiments and animals. G-CSF treatment significantly inhibited spontaneous and drug-induced apoptosis of resting *wt* granulocytes after treatment with etoposide or doxorubicin ($P < 0.035$ or lower) but had no statistically significant impact on cell death induced by cis-platin or FasL. In granulocytes from peritoneal exudate only spontaneous death was significantly delayed by G-CSF treatment ($P < 0.004$).

G-CSF Inhibits Spontaneous and Drug-induced Granulocyte Apoptosis but Has No Influence on FasL-induced Cell Death. Cytokines can influence the expression levels and activity of pro as well as antiapoptotic members of the Bcl-2 family (5–7). Therefore, we investigated the effects of G-CSF on granulocyte survival in culture. Pretreatment of bone marrow–derived granulocytes with G-CSF delayed spontaneous cell death (Fig. 4 a), as well as apoptosis induced by the anticancer drugs etoposide (Fig. 4 b) and doxorubicin (Fig. 4 c), but had no effect on cis-platin– or FasL-induced cell death (Fig. 4, d and e). Similarly, G-CSF pretreatment of peritoneal exudate granulocytes significantly delayed spontaneous apoptosis (Fig. 4 f), but did not impair FasL-induced killing (Fig 4 g). Although G-CSF protected bone marrow granulocytes against etoposide-induced apoptosis, it provided almost no protection to peritoneal exudate granulocytes, perhaps due to their rapid kinetics of spontaneous death under these conditions (Fig. 4 h).

Since treatment with G-CSF increases the expression of the antiapoptotic Bcl-2 family members A1 and Mcl-1 in myeloid cells (5, 15), we investigated whether G-CSF can also regulate other Bcl-2 family members. Granulocytes from bone marrow were cultured for 0, 4, or 16 h in the presence or absence of G-CSF. Expression of Bcl-2, Bcl-X_L, and Bax within the Gr-1^+ population was determined by cytoplasmic immunofluorescence staining of fixed, permeabilized cells and flow cytometric analysis. Freshly isolated granulocytes expressed low level of Bcl-2, Bcl-X_L, and Bax, and G-CSF did not notably alter their expression levels (data not shown). As in lymphoid cells (26), levels of Bim were below the threshold of detection by immunoflu-

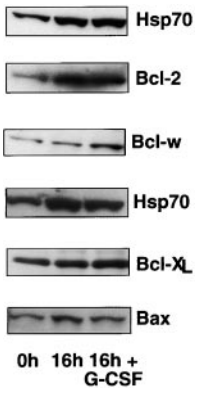

Figure 5. Expression of Bcl-2 family members in granulocytes. Immunoblot analysis of Bcl-2, Bcl-w, Bcl-X_L, and Bax expression in extracts from freshly isolated granulocytes (0 h) or granulocytes after culture for 16 h in medium alone or medium supplemented with 100 ng/ml G-CSF. Filters had to be exposed between 10 min (Bax, Bcl-X_L, and Bcl-w) and 60 min (Bcl-2) to detect signals specific for these Bcl-2 family members. Membranes were stripped and re-probed with an anti-Hsp70 Ab as a loading control.

orescence staining (data not shown). Immunoblotting confirmed the results obtained by flow cytometric analysis and also revealed that granulocytes express low levels of the antiapoptotic protein Bcl-w (Fig. 5).

Collectively, these results show that G-CSF can inhibit spontaneous and chemotherapeutic drug–induced apoptosis but has no effect on FasL-mediated cell killing. Although G-CSF may well mediate survival by inducing expression of Bcl-2 family members such as A1 or Mcl-1 (5, 15), it does not noticeably affect the levels of Bcl-2, Bcl-X_L, Bax, Bcl-w, or Bim in mouse granulocytes.

The Stress-activated Kinase p38 MAPK Promotes Granulocyte Survival. Stress-activated kinases such as p38 MAPK or JNK have been implicated in the control of apoptosis (21, 40–42). It has been reported that p38 MAPK is constitutively activated in granulocytes and that this promotes the death induced by cytokine withdrawal, since synthetic inhibitors of p38 MAPK delayed spontaneous but not anti-CD95 Ab–induced cell death (11, 12). We first investigated which apoptotic stimuli could activate p38 MAPK or the related kinase JNK. Sorted granulocytes from *wt* and *lpr* mice were cultured without supporting cytokines, with soluble FasL, multimerized FasL, or etoposide. Analysis of the cell extracts using an Ab recognizing the phosphorylated form of p38 MAPK confirmed its constitutive activation in granulocytes (Fig. 6 a). p38 MAPK activity was augmented in *wt* cells treated with multimerized but not soluble FasL. As expected, no such effect was observed in cells derived from CD95-deficient *lpr* mice (Fig. 6 b), but both *wt* and *lpr* granulocytes responded to etoposide with enhanced p38 MAPK activity (Fig. 6, a and b). The membranes were also probed with Abs specific for activated forms of JNK1 and JNK2, or the related MAPKs ERK1 and ERK2. No basal activation of JNK could be detected and application of multimerized FasL failed to induce phosphorylation of JNK or ERK. In contrast, phosphorylated JNK and ERK were readily detected in lysates from spleen cells treated with anisomycin or PMA plus ionomycin (data not shown).

To investigate the significance of p38 MAPK activity in granulocyte apoptosis, we tested whether the specific synthetic inhibitors of p38 MAPK, SB203850 or SB202130, could modulate spontaneous or FasL-induced cell death. These inhibitors have been shown to specifically inhibit

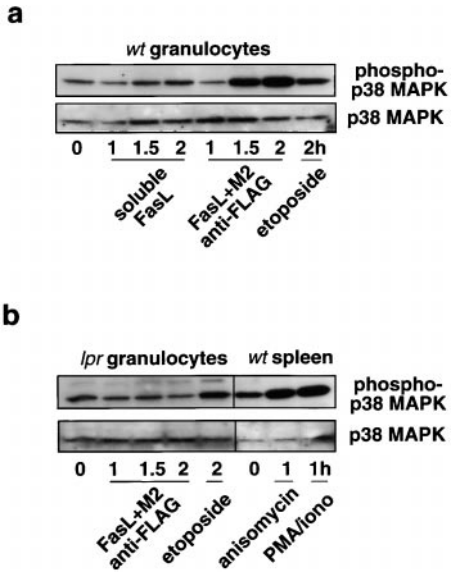

Figure 6. Multimerized FasL enhances p38 MAPK activity in granulocytes. Sorted granulocytes from bone marrow of (a) *wt* and (b) *lpr* mice were treated with soluble or multimerized FasL (100 ng/ml), with etoposide (50 μg/ml), or were left untreated. (b) Spleen cells from *wt* mice stimulated with 10 μg/ml anisomycin or 100 ng/ml PMA plus 1 μg/ml ionomycin served as a positive control for p38 MAPK activation. p38 MAPK activity was determined by immunoblotting of cell extracts using an Ab specific for phosphorylated p38 MAPK. Equal protein loading was confirmed by reprobing membranes with an Ab recognizing p38 MAPK irrespective of its status of phosphorylation.

p38 MAPK α and β isoforms but not other related kinases such as p38 MAPK δ or γ, JNKs, or ERKs (43). We first analyzed their effect on spontaneous death of granulocytes after 24 h (Fig. 7 a) and 48 h (not shown) in culture using as controls cells treated with the solvent DMSO or the MEK-1 inhibitor PD98059. Contrary to previous reports (11, 12), the two p38 MAPK inhibitors did not delay but drastically accelerated spontaneous death of granulocytes in culture. Whereas these inhibitors enhanced granulocyte killing in a dose- and time-dependent manner, preincubation of cells with G-CSF abolished this effect. DMSO or PD98059, which specifically targets the ERK pathway, had no significant effect on granulocyte survival (Fig 7 a, and data not shown).

To investigate whether G-CSF exerts its antiapoptotic effect by activating the p38 MAPK pathway, we analyzed lysates from sorted granulocytes treated with G-CSF in vitro for p38 MAPK activity. Immunoblotting demonstrated that G-CSF did not augment the basal p38 MAPK activity but, as reported previously, for human granulocytes (44) it did significantly elevate ERK phosphorylation (Fig. 7 b). Furthermore, a time course experiment demonstrated that G-CSF could prevent p38 MAPK inhibitor–induced apoptosis, whether applied 30 min before, simultaneously, or even 30 min after addition of SB203580 (Fig. 7 c) or SB202190 (data not shown).

Collectively, these data indicate that G-CSF inhibits p38 MAPK inhibitor–induced granulocyte killing by activating

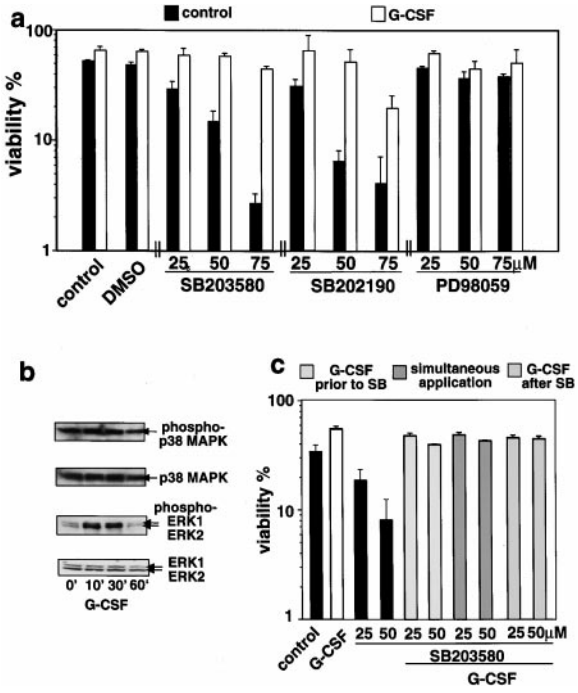

Figure 7. Inhibition of p38 MAPK accelerates death of granulocytes. (a) Sorted granulocytes from bone marrow of *wt* mice were cultured in the absence or presence of human G-CSF (100 ng/ml), which was added 30 min before treatment with either DMSO (0.25%), the p38 MAPK inhibitors SB203580 or SB202190, or the MEK-1 inhibitor PD98059. Cell viability was assessed after 24 h in culture. Both p38 MAPK inhibitors significantly accelerated spontaneous apoptosis of resting *wt* granulocytes ($P <$ 0.002 or lower). (b) Sorted granulocytes from bone marrow of *wt* mice were treated with G-CSF (100 ng/ml) for the time points indicated or were left untreated. p38 MAPK or ERK1 and ERK2 activity was determined by immunoblotting of cell extracts using an Ab specific for phosphorylated p38 MAPK or phosphorylated ERK1 and ERK2, respectively. Equal protein loading was confirmed by reprobing membranes with an Ab recognizing p38 MAPK or ERK1 and ERK2 irrespective of their status of phosphorylation. (c) Sorted granulocytes from bone marrow of *wt* mice were cultured in the absence or presence of human G-CSF (100 ng/ml), which was added 30 min before, simultaneously with, or 30 min after application of the p38 MAPK inhibitor SB203580 (SB). Cell viability was assessed after 48 h in culture. Data shown represent arithmetic means ± SD of three to five animals and two independent experiments.

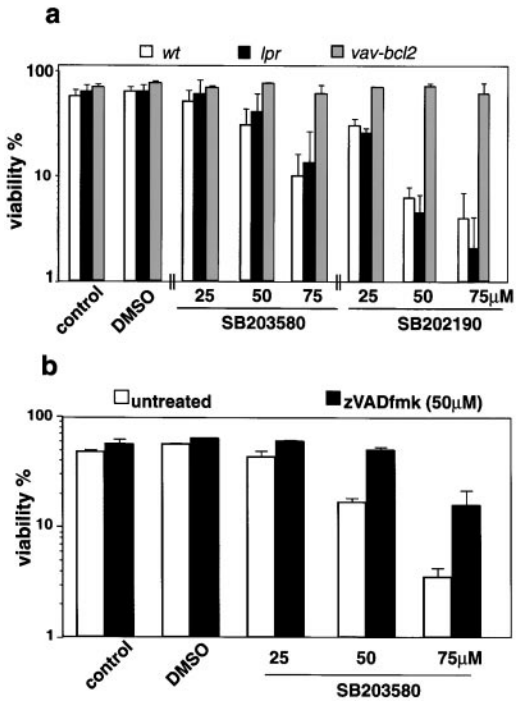

Figure 8. Cell death induced by inhibition of p38 MAPK is mediated by a Bcl-2–inhibitable and caspase-dependent apoptotic pathway. (a) Sorted granulocytes from bone marrow of *wt*, *lpr*, or *vav-bcl-2* transgenic mice were cultured with either DMSO (0.25%) or the p38 MAPK inhibitors SB203580 or SB202190. Cell viability was assessed after 24 h in culture. (b) Sorted granulocytes from *wt* mice were cultured in the absence or presence of the caspase inhibitor zVADfmk (50 μM), which was added 30 min before treatment with either DMSO (0.25%) or the p38 MAPK inhibitor SB203580. Cell viability was assessed after 24 h in culture as described in the legend to Fig. 2. Data shown represent arithmetic means ± SD of two to four animals.

a survival pathway that can substitute for p38 MAPK signaling. The activation of the ERK pathway by G-CSF does not seem to be essential for mediating its antiapoptotic effect, since application of the MEK-1 inhibitor PD98059 did not block G-CSF–induced inhibition of spontaneous granulocyte death (Fig. 7 a, and data not shown).

The death of granulocytes induced by the p38 MAPK inhibitors occurred by apoptosis. Dying cells displayed classical apoptotic morphology, including condensed chromatin and phosphatidylserine exposure (not shown), and cell killing could be inhibited by overexpression of Bcl-2 (Fig. 8 a) or the broad spectrum caspase inhibitor zVADfmk (Fig. 8 b). The p38 MAPK inhibitor–mediated killing was independent of the FasL/CD95 system since *lpr* granulocytes were as sensitive as *wt* cells to these inhibitors (Fig. 8 a).

Discussion

Granulocytes are short-lived in vivo and readily undergo spontaneous as well as damage-induced apoptosis in culture. To clarify the control of their death, we have examined the role of the pathways involving FasL/CD95, G-CSF, p38 MAPK, and the Bcl-2 protein family.

Experiments with lymphocytes have shown that Abs to CD95 do not reliably mimic the physiological ligand for CD95 and that high concentrations of soluble trimeric FasL can even antagonize the proapoptotic activity of membrane-bound FasL, presumably by restricting the extent of CD95 aggregation (23, 24, 28). To investigate whether these findings are also relevant to a different cell type, we compared the death-inducing properties of soluble, multimerized, and membrane-bound FasL on granulocytes (Fig. 1 c). In accordance with the studies on lymphocytes (23, 24, 28), killing by FasL required multimerization of the receptor, a state not achievable by application of soluble FasL. Our findings may indicate that FasL-induced apoptosis in all cell types requires receptor aggregation and not simply trimerization. Soluble FasL has been reported to trigger chemotaxis in granulocytes (45, 46). Hence, trimerization

or multimerization of CD95 may elicit qualitatively different responses in certain cell types.

It is controversial whether CD95-mediated killing can be inhibited by antiapoptotic members of the Bcl-2 family (27, 31–33, 36). In addition, the proapoptotic Bcl-2 family member Bid was reported to connect CD95 signaling with the Bcl-2 family. This pathway appears to be dispensable for CD95 killing of lymphocytes but may have a more significant role in other cell types, such as hepatocytes (47). We analyzed whether Bcl-2 overexpression affected FasL-induced apoptosis by comparing *wt*, mutant *lpr*, and *vav-bcl-2* transgenic granulocytes. The apoptosis induced by either multimerized recombinant FasL or membrane-bound FasL was unaffected by Bcl-2 overexpression (Fig. 2, d, f, and h). These findings are consistent with observations on thymocytes, resting T cells, and activated T and B cells, in all of which *bcl-2* transgene expression also failed to protect against CD95-induced apoptosis (23, 27, 35, 48). In addition, evidence was provided recently that hepatocytes expressing a *bcl-2* transgene retain normal sensitivity to the cytotoxic effects of multimerized FasL (23). Others have reported that killing of hepatocytes induced in vivo by anti-CD95 Abs can be inhibited by Bcl-2 (32, 33). However, it is unclear whether anti-CD95 Abs kill hepatocytes directly or by an indirect mechanism that also involves IFN-γ, as is the case when mice are injected with soluble FasL and *Propionibacterium acnes* (49). Collectively, these results indicate that Bcl-2 does not interfere with FasL-induced apoptosis in diverse cell types.

In contrast to FasL-induced cell killing, both spontaneous and drug-induced apoptosis were significantly reduced in granulocytes from *vav-bcl-2* transgenic mice (Fig. 3, a, b, d, and e). The protective effect of Bcl-2 on spontaneous apoptosis of granulocytes was previously demonstrated in *MRP-8-bcl-2* transgenic mice, but its impact on FasL- or drug-induced cell death was not addressed in that study (50). Our evidence that Bcl-2 can inhibit the apoptosis induced by etoposide, cis-platin, or doxorubicin is consistent with the observation that Bcl-2 protects bone marrow cells against drug-induced myelosuppression (51). However, a caveat for attempts to use overexpression of Bcl-2 to rescue bone marrow cells from chemotherapy-induced cytotoxicity is that the Bcl-2–based protection was limited and most cells did eventually die (Fig. 3, and data not shown).

Previous reports implicated autocrine and/or paracrine FasL/CD95 signaling as a cause of the spontaneous death of granulocytes and the chemotherapeutic drug–induced death of certain tumor cells (9, 17, 18, 39). However, our study demonstrated that granulocytes from CD95-deficient *lpr* mice exhibit normal sensitivity to both spontaneous and drug-induced apoptosis (Fig. 3, c and f). Consistent with our results, other reports showed that *lpr* mice have normal numbers of granulocytes and myeloid progenitors with colony-forming potential (19, 20). Moreover, blocking CD95 signaling by application of neutralizing Abs to CD95, or by elimination of caspase-8 or FADD activity, did not alter apoptosis of lymphocytes or mouse embryonic fibroblasts induced by chemotherapeutic drugs or γ-radiation (34, 52–54).

G-CSF promotes maturation of myeloid precursors and can extend granulocyte life span (1, 4). It has been reported that G-CSF can inhibit death induced by anti-CD95 Ab treatment in human neutrophils (9). There is controversy as to whether G-CSF can inhibit drug-induced apoptosis of granulocytes. One report showed that G-CSF attenuates the cytotoxic effect of etoposide on myeloid cells in vivo (55), but another claimed that G-CSF enhanced Ara-C–induced apoptosis of myeloid leukemia cells (56). Our results show that G-CSF can delay spontaneous death and provide significant protection against etoposide- or doxorubicin-mediated killing but has no effect on FasL-induced apoptosis (Fig. 4, a–e). Mobilization of granulocytes to a site of inflammation in vivo did not alter their sensitivity to either etoposide or FasL (Fig. 4, f and g). These observations conflict with reports that priming of granulocytes with LPS, GM-CSF, or G-CSF interferes with anti-CD95 Ab–induced apoptosis (9, 18). Although this may indicate a difference between human peripheral blood granulocytes and murine bone marrow–derived granulocytes, we prefer the explanation that this discrepancy is due to the fact that anti-CD95 Abs do not reliably mimic the physiological ligand for CD95 (23).

Inflammatory cytokines or LPS can influence survival of granulocytes by increasing the expression of antiapoptotic members of the Bcl-2 family such as A1 or Mcl-1 (5–7, 15). We observed low level expression of Bcl-2, Bcl-X_L, and Bcl-w in granulocytes, but this was not increased by treatment with G-CSF (Fig. 5). Although no granulocytic abnormalities have been reported in Bcl-2–, Bcl-w–, or Bcl-X_L–deficient mice (57–59), all of these proteins contribute in a redundant manner to the regulation of granulocyte survival since A1-deficiency enhances granulocyte death only to a limited extent (16). It would be interesting to investigate the impact of deficiency for both A1 plus Bcl-2, Bcl-w, or Bcl-X_L.

G-CSF might also downregulate the expression or activity of proapoptotic Bcl-2 family members. The delayed apoptosis of granulocytes from patients with inflammatory diseases has been ascribed to reduced levels of Bax because its levels in human granulocytes rose during culture in simple medium, and the rise was blocked by inflammatory cytokines such as IL-3, G-CSF, or GM-CSF (60). We observed that Bax was constitutively expressed in cultured mouse granulocytes, but its level was not influenced by G-CSF (Fig. 5). Although we detected only very low levels of Bim by a highly sensitive immunoprecipitation/Western blotting technique (data not shown), it must play a role in granulocyte apoptosis because Bim-deficient mice have a threefold excess of granulocytes (61). This is consistent with the finding that Bim, although expressed at very low levels, is an essential inducer of lymphocyte apoptosis (61). It appears that the activity of some proapoptotic Bcl-2 family members is regulated primarily at a posttranslational level. For example, Bim is inactivated by sequestration to microtubules in healthy cells but is released after cytokine withdrawal to unleash its proapoptotic function (62).

The stress-activated kinase p38 MAPK has been shown

to activate transcription factors such as myocyte enhancer factor 2 or activating transcription factor 2 (21), and has also been implicated in the activation of nuclear factor (NF)-κB signaling (63). p38 MAPK has also been reported to act as an activator of cell death induced by γ-radiation, growth factor deprivation, or B cell receptor cross-linking (40, 64–66). Conflicting results have been published regarding the involvement of p38 MAPK in the regulation of granulocyte apoptosis. One study reported that stress-induced but not spontaneous apoptosis depends on p38 MAPK activity (12), whereas another report implicated p38 MAPK activation in spontaneous granulocyte death (11). Neither study found any evidence for activation of p38 MAPK by anti-CD95 Ab treatment (11, 12). In contrast, we found that multimerized FasL enhanced p38 MAPK activity in *wt* but not *lpr* granulocytes (Fig. 6). Unexpectedly, we found that application of specific inhibitors of p38 MAPK accelerated spontaneous death of granulocytes in vitro (Fig. 7 a). The cytotoxicity resulting from p38 MAPK inhibition induced apoptotic death, since it was prevented by application of zVADfmk or overexpression of Bcl-2 (Fig. 8, a and b). Similarly, preincubation of granulocytes with G-CSF also prevented the cell death induced by inhibition of p38 MAPK (Fig. 7 a). Although human granulocytes derived from peripheral blood may respond differently to p38 MAPK inhibition than murine granulocytes derived from bone marrow, we conclude that p38 MAPK activation is part of a general stress response that mediates survival in granulocytes, and that G-CSF signaling can substitute for p38 MAPK, probably by inducing A1 or Mcl-1 expression. Given the observation that p38 MAPK mediates NF-κB activation in LPS-stimulated granulocytes (63), it is conceivable that this might lead to the transcriptional activation of certain antiapoptotic Bcl-2 family members that might mediate the antiapoptotic effect of LPS. This model would be consistent with the observation of Rel/NF-κB–mediated transcriptional activation of A1 in lymphocytes (67). In further agreement with our findings, the p38 MAPK inhibitor SB202190 was recently shown to enhance UV- and anti-CD95–induced apoptosis in Jurkat and HeLa cells (68), and p38 MAPKα$^{-/-}$ embryonic stem cells were shown to have normal sensitivity to treatment with staurosporine or doxorubicin (69). Taken together, these results make an essential role for p38 MAPK in the mediation of apoptosis unlikely but rather indicate that it has a prosurvival function. p38 MAPK might therefore be a useful target in the treatment of inflammatory diseases to prevent accumulation of granulocytes.

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