Granulocyte Macrophage Colony-stimulating Factor Signaling and Proteasome Inhibition Delay Neutrophil Apoptosis by Increasing the Stability of Mcl-1*

Mathieu Derouet, Luke Thomas, Andrew Cross, Robert J. Moots§, and Steven W. Edwards§

From the School of Biological Sciences, Biosciences Building and 3Department of Medicine, University of Liverpool, Liverpool L69 7ZB, United Kingdom

Human neutrophils normally have a very short half-life and die by apoptosis. Cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) can delay this apoptosis via increases in the cellular levels of Mcl-1, an anti-apoptotic protein of the Bcl-2 family with a rapid turnover rate. Here we have shown that inhibition of the proteasome (a) decreases the rate of Mcl-1 turnover within neutrophils and (b) significantly delays apoptosis. This led us to determine whether GM-CSF could enhance neutrophil survival by altering the rate of Mcl-1 turnover. Addition of GM-CSF to neutrophils enhanced Mcl-1 stability and delayed apoptosis by signaling pathways requiring PI3K/Akt and p44/42 Erk/Mek, because inhibitors of these pathways completely abrogated the GM-CSF-mediated effect on both Mcl-1 stability and apoptosis delay. Conversely, induction of Mcl-1 hyperphosphorylation by the phosphatase inhibitor, okadaic acid, significantly accelerated both Mcl-1 turnover and apoptosis. Neither the calpain inhibitor, carboxybenzoyl-valinyl-phenylalaninal, nor the pan caspase inhibitor, benzylxycarbonyl-VAD-fluoromethylketone, had any effect on Mcl-1 stability under these conditions. These observations indicate that profound changes in the rate of neutrophil apoptosis following cytokine signaling occur via dynamic changes in the rate of Mcl-1 turnover via the proteasome.

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§ To whom correspondence should be addressed. Tel.: 44-151-795-4425; Fax: 44-51-795-4404; E-mail: S.W.Edwards@liv.ac.uk.

1 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Z-VAD-fmk, benzylxycarbonyl-VAD-fluoromethyl ketone; FITC, fluorescein isothiocyanate; PI3K, phosphatidylinositol 3-kinase.
of turnover and, in parallel, considerably accelerated neutrophil apoptosis. These data show, for the first time, that cytokine-mediated neutrophil survival can be controlled by changes in the turnover rate of a normally labile molecule that plays a critical and dynamic role in cell survival.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium and fetal calf serum were from Invitrogen. Anti-human Mcl-1 monoclonal antibody (13656E) was from BD Pharmingen. Okadaic acid, cypermethrin, phosphatase inhibitor mixture set II (imidazole, sodium fluoride, sodium molybdate, sodium orthovanadate, sodium tartrate dihydrate), MG-132, PD98059, SB202190, H-89, GF20900X, wortmannin, ALLN (N-acetyl-leucinyl-leucinyl-norleucinal), MDL 28170 (carbobenzoxyl-valinyl-phenylalaninal), lactacystin, and Z-VAD-fmk were from Calbiochem. Cycloheximide, pooled human AB serum, and sheep anti-mouse IgG were from Sigma-Aldrich. GM-CSF was from Roche Applied Science. FITC-annexin V was from BIOSOURCE Int. Anti-phospho p44/42 Thr^202/Tyr^204 (ERK1/2), anti-phospho p38 MAPK Thr^180/Tyr^182, anti-phospho Akt Ser^463 were from Cell Signaling. Donkey anti-rabbit IgG and the ECL detection kit were from Amersham Biosciences. Donkey anti-goat IgG was from Santa Cruz Biotechnology Inc. Polymorphprep was from Robbins Scientific (Europe) Ltd., Solihull, UK.

Isolation, Purification, and Incubation of Neutrophiles—Neutrophils were isolated from heparinized venous blood from healthy volunteers by sedimentation to remove contaminating erythrocytes, cells were resuspended in RPMI 1640 medium supplemented with 5% pooled human AB serum at 5 × 10^6/ml. Culture was at 37 °C with gentle agitation. Purity and viability were routinely above 95% as assessed by May-Grünwald-Giemsa staining and trypan blue exclusion, respectively. GM-CSF was added at 50 ng/ml. The following additions were also made as indicated in the text: cycloheximide, 10 μg/ml; MG-132, 50 μm; okadaic acid, 1 μm; cypermethrin, 1 μm; phosphatase inhibitor mixture II, 1:100 dilution; PD 98059, 50 μM; SB202190, 1 μM; wortmannin, 50 nM; H-89, 5 μM; LY294002, 10 μg/ml; GF203109X, 200 μM; ALLN, 100 μM; carbobenzoxyl-valinyl-phenylalaninal (MDL 2810), 1 μM; Z-VAD-fmk, 50 μM; lactacystin, 40 μM.

Morphological Estimation of Apoptosis—Following culture, a 20-μl aliquot of suspension was made up to 200 μl with RPMI 1640, and cells were cytocentrifuged using a Shandon Cytospin3 (Runcorn, Cheshire, UK). Romanowsky staining of cytospins allowed apoptosis to be scored by morphology as described in Ref. 22. This method correlates well with other markers of apoptosis (23).

Annexin V-FITC Staining—Neutrophils (10^6) were removed from culture and resuspended in Annexin V binding buffer (10 mM Heps, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), before the addition of annexin V-FITC (at a 1:100 dilution). After 10 min in the dark, cells were pelleted at 400 × g and resuspended in Annexin V binding buffer before analysis by flow cytometry using a Coulter-EpicsAltra flow cytometer. 10000 cells/sample were analyzed.

Western Analysis—Following culture, 10^6 cells were rapidly lysed in boiling reducing SDS-PAGE sample buffer containing aprotonin (20 μg/ml), leupeptin (20 μg/ml), pepstatin (10 μg/ml), phenylmethylsulfonyl fluoride (400 μg/ml), phosphatase inhibitor set II (1:100 dilution), immediately boiled for 5 min with occasional vortexing, and stored at −80 °C until use. SDS-PAGE and electrotransfer to polyvinylidene difluoride membranes were performed as described (24). Primary antibodies used were: Mcl-1 (13656E), anti-phospho p44/42 (ERK), anti-phospho p38 MAPK, anti-phospho Akt. The horseradish peroxidase-conjugated secondary antisera used were donkey anti-rabbit IgG and donkey anti-goat IgG. Bound antibodies were detected using the ECL system. Densitometry on carefully exposed blots (to avoid film saturation) was performed with Image 1.44 VDM software (NIH). Ponceau S-stained actin on membranes after electrotransfer was measured to control for loading of neutrophil samples.

Statistics—Data sets were analyzed using the paired Student’s t test.

RESULTS

Effects of Proteasome Inhibition on Mcl-1 Levels and Apoptosis—Previous work has shown that Mcl-1 has a short half-life within cells because PEST sequences within the protein may target it for proteolysis via the proteasome. We therefore tested whether inhibition of proteasome function had any effect on the cellular levels of Mcl-1 and apoptosis in human neutrophils. Neutrophils incubated for 3 h (in the absence of any added agents) had greatly increased levels of Mcl-1 compared with levels detected in freshly isolated cells (Fig. 1). Levels of Mcl-1 were depleted even further in cultures incubated for 3 h in the presence of cycloheximide to block de novo protein biosynthesis, indicating the rapid turnover of the protein when biosynthesis is inhibited. The addition of GM-CSF maintained cellular levels of this protein, but remarkably, incubation for 3 h with the proteasome inhibitor MG-132 resulted in levels of Mcl-1 that were significantly greater than those detected in freshly isolated cells. No changes in the levels of Bax or actin were observed in these experiments, indicating that these latter two proteins were relatively stable within neutrophils. These experiments confirm the idea that the normally short half-life of Mcl-1 is because of its rapid turnover via the proteasome.

In view of the proposal that neutrophil cell death and survival is critically dependent on the cellular levels of Mcl-1 (4, 10, 12), we then tested whether manipulation of the levels of this protein by altering its turnover rate had any effect on neutrophil apoptosis. Neutrophils incubated with these same agents were tested in parallel experiments for the appearance of apoptotic features as assessed by annexin binding or morphology. Cultures of freshly isolated neutrophils contained no apoptotic cells (data not shown), but by 8 h of incubation in the absence of any exogenous agents, over 40% of the cells exhibited apoptotic features as determined by annexin binding or morphology. The addition of GM-CSF caused a significant increase in apoptosis (Fig. 2A) or by morphology (Fig. 2B and C). When incubation was blocked by the addition of cycloheximide, apoptosis was significantly increased to over 75% (±10%, n = 5, p < 0.01) as has been reported previously (25–27). The addition of MG-132 also prevented activation of caspase-3 (data not shown). Thus, prevention of Mcl-1 turnover by inhibition of proteasome function (Fig. 1) had a parallel effect on delaying neutrophil apoptosis.

Effects of GM-CSF on Rates of Mcl-1 Turnover—We have previously shown that GM-CSF can stimulate MCL-1 transcription and increase cellular mRNA levels (14, 19). However, in view of the striking increases in Mcl-1 levels observed after inhibition of proteasome function, we decided to determine
whether GM-CSF could increase cellular levels of Mcl-1 not by increasing de novo biosynthesis but instead by altering its stability and turnover rate. Neutrophils were blocked in de novo biosynthesis by incubation with cycloheximide in the absence and presence of GM-CSF; at time intervals, levels of Mcl-1 were determined by Western blotting. In the absence of GM-CSF, blocking de novo biosynthesis resulted in a rapid decrease in cellular levels of Mcl-1 because of its high turnover rate (Fig. 3), estimated to be \( \frac{1}{2} \) h in these experiments. In contrast, in the presence of GM-CSF the stability of Mcl-1 was dramatically increased. These experiments clearly indicate that GM-CSF treatment can increase Mcl-1 protein levels by decreasing its rate of turnover via the proteasome.

Signaling Pathways Regulating GM-CSF Changes in Mcl-1 Levels—Having shown that GM-CSF treatment greatly increased Mcl-1 stability in human neutrophils, it was then necessary to determine the signaling pathways that controlled this phenomenon. Several intracellular signaling systems have been implicated in mediating the effects of GM-CSF on apoptosis delay in neutrophils (1, 28–32). Levels of activated Erk1/2, p38 MAPK, and Akt were low or undetectable in resting neutrophils (Fig. 4) but rapidly increased following GM-CSF exposure. Maximal activation of Erk1/2 and Akt occurred within 3 min of addition of cytokine, whereas maximal activation of p38 MAPK occurred by 7 min (Fig. 4A). Levels of activated Erk1/2 and Akt were maintained for about 30 min but then declined to basal levels by 5 h after stimulation (Fig. 4B). However, levels of activated p38 MAPK showed more complex changes (Fig. 4C). After initial stimulation, levels of activated p38 MAPK decreased but transiently increased again by \( \frac{1}{2} \) h after GM-CSF treatment and again by 5 h poststimulation.
This pattern of transient activation was consistently seen in seven separate experiments with neutrophils isolated from different donors. Control experiments indicated that p38-MAPK became slowly activated by 4–5 h culture in untreated cells, even in the absence of GM-CSF. However, the transient activation seen at 2–3 h was only seen following GM-CSF treatment. These experiments confirm a number of putative kinase signaling pathways activated by GM-CSF; hence it was necessary to determine whether any of these played a role in regulating Mcl-1 stability.

Neutrophils were therefore treated with a panel of signaling inhibitors to test for their ability to affect GM-CSF signaling of enhanced Mcl-1 stability and apoptosis. Cells were incubated with cycloheximide to block protein biosynthesis in the presence and absence of GM-CSF and the inhibitors. After 2 h of incubation with cycloheximide alone, cellular levels of Mcl-1 had decreased to ~40% of the time 0 levels, again indicating rapid turnover of the protein in the absence of de novo biosynthesis (Fig. 5, A and B). In the presence of GM-CSF, the turnover of Mcl-1 was slowed; when proteasome activity was blocked with MG-132, protein levels were increased further, indicating negligible turnover. Blocking the activity of p38-MAPK had no effect on the GM-CSF-dependent increase in Mcl-1 stability. In contrast, blocking the activities of Erk/Mek and PI3K with PD98059 and wortmannin (or LY294002, data not shown), respectively, completely abrogated the GM-CSF-mediated increase in Mcl-1 stability. Thus, in the presence of these latter inhibitors, the turnover rate of Mcl-1 was identical to that measured in cycloheximide-only treated cultures. The stability changes in Mcl-1 following GM-CSF exposure require signals generated via Erk/Mek and PI3K/Akt. No effects on Mcl-1 turnover rate were observed after the addition of I89 (an inhibitor of protein kinase A) or GF209309X (an inhibitor of protein kinase C) (data not shown).

Parallel changes in rates of neutrophil apoptosis were observed in the presence of GM-CSF and these signaling inhibitors. After 8 h of incubation in the presence of cycloheximide, ~80% of the cells were apoptotic as assessed by annexin V binding or morphology (Fig. 5C). Even when cells were blocked in protein biosynthesis, GM-CSF could still significantly delay apoptosis; when proteasome function was inhibited (with MG-132), the protective effect of GM-CSF was even greater. The p38-MAPK inhibitor SB202190 had little effect on this GM-CSF-regulated effect on apoptosis. In contrast, PD98059 and wortmannin (or LY294002, data not shown) completely abrogated the protective effects of GM-CSF on neutrophil apoptosis in these cycloheximide-treated cells.

It was then necessary to determine whether other proteolytic processes known to function in neutrophils had any effect on the turnover of Mcl-1. The pan caspase inhibitor Z-FAD-fmk had no effect on Mcl-1 turnover when cells were incubated with cycloheximide in the absence or presence of GM-CSF (Fig. 6, A and B). Similarly, the cell-permeable calpain inhibitor carbo- benzoxyl-valinyl-phenylalinalenil (33) had no effect on Mcl-1 turnover under these experimental conditions. However, ALLN (known to inhibit calpains and the proteasome) (34, 35) and the proteasome inhibitors lactacystin (36) and MG-132 all showed remarkable similarities in their ability to prevent Mcl-1 turnover. Taken together, these observations indicate a role for the proteasome, but not calpains nor caspases, in regulating Mcl-1 turnover in neutrophils.

Effects of Protein Phosphorylation on Mcl-1 Stability and Turnover—We then sought to determine whether hyperphosphorylation of proteins by use of phosphatase inhibitors could have any effect on the GM-CSF-mediated increase in Mcl-1 stability and decrease in apoptosis. Neutrophils were incubated with cycloheximide in the absence and presence of GM-CSF together with a panel of broad range or specific phosphatase inhibitors. The inclusion of cypemethrin had little effect on Mcl-1 stability, but the broad range phosphatase inhibitors and, particularly, okadaic acid treatment resulted in a marked decrease in cellular levels of Mcl-1 in cells blocked in de novo synthesis, indicating that this phosphatase inhibitor accelerated Mcl-1 turnover (Fig. 7A). Indeed, in the presence of okadaic acid, turnover of Mcl-1 was even higher than that observed in cells treated with cycloheximide alone. Thus, okadaic acid not only negated the protective effect of GM-CSF, it accelerated the endogenous turnover rate of Mcl-1 even further. Okadaic acid also resulted in a shift in the mobility of Mcl-1 on SDS-PAGE, indicating its de novo rapid turnover of the protein in the absence of de novo biosynthesis.

Fig. 5. The GM-CSF-mediated stability increase in Mcl-1 is signaled through Erk/Mek and PI3K/Akt. Neutrophils were incubated for 0 or 2 h in the presence of cycloheximide (10 μg/ml). GM-CSF (50 ng/ml) was added as indicated in the absence or presence of MG-132 (MG, 50 μM), PD98059 (PD, 50 μM), SB202190 (SB, 1 μM), or wortmannin (Wort, 20 nM). Mcl-1 levels were then measured by Western blotting. A, representative Western blot. B, mean relative Mcl-1 levels after 2 h of incubation (± S.D.) measured by densitometry (the value of freshly isolated cells taken as 100%), n = 5. *p < 0.02 compared with GM-CSF plus cycloheximide alone. Levels of actin (Ponceau-stained filter) are also shown. C, neutrophils were incubated in the absence (Control) and presence of cycloheximide and GM-CSF as indicated. After 8 h of incubation, apoptosis was measured by either FITC-annexin V binding (□) or morphology (□). Values shown are mean (±S.D.), n = 5. *p < 0.02 of indicated data sets.
increased phosphorylation (Fig. 7B). These results clearly show that hyperphosphorylation of Mcl-1 accelerates its normally high turnover rate.

In parallel experiments, the broad range phosphatase inhibitors and okadaic acid treatment significantly blocked the GM-CSF-mediated decrease in apoptosis in cells blocked in de novo biosynthesis; levels of apoptosis were even greater than those observed during incubation with cycloheximide alone (Fig. 7C). These experiments clearly demonstrate that hyperphosphorylation of Mcl-1 accelerates its rate of turnover and greatly increases the rate at which neutrophils undergo apoptosis.

Regulation of Mcl-1 turnover in neutrophils (Fig. 8A) shows that Mcl-1 turnover in cycloheximide-treated neutrophils is slowed by GM-CSF, virtually completely blocked by proteasome inhibition, and greatly accelerated after inhibition of the phosphatases PP2A/PP1 by okadaic acid. Fig. 8B shows that the increased stability of Mcl-1 induced by GM-CSF is unaffected by SB202190 but completely blocked by inhibition of Erk/Mek and PI3K by PD98059 and LY294002, respectively.

**DISCUSSION**

In this report, we have shown for the first time that the rate at which neutrophils undergo apoptosis is critically dependent upon the rate of turnover of Mcl-1, an anti-apoptotic protein of the Bcl-2 family. We have also shown, for the first time, that the cytokine GM-CSF can result in dramatic increases in cellular levels of a protein, not by altering its rate of de novo synthesis but rather by increasing its stability and decreasing its rate of turnover. Most studies to date on the effects of cytokines on immune cells have focused upon activation of transcription and modification of the activity of pre-existing proteins, for example by phosphorylation. To our knowledge, this is the first report to show a cytokine-mediated change in immune cell function by increasing the stability of a survival protein. We have also shown that the PI3K/Akt and Mek/Erk signaling pathways are intimately involved in this GM-CSF-mediated effect on Mcl-1 stability and that Mcl-1 hyperphos-
GM-CSF Increases Mcl-1 Stability

Fig. 8. Manipulation of Mcl-1 stability. Neutrophils were incubated with cycloheximide (10 µg/ml) plus the following agents. A, MG-132 (10 µM); GM-CSF (50 ng/ml); cycloheximide only (○); okadaic acid (■ 1 µM); B, GM-CSF only (○); GM-CSF + SB202190 (○ 1 µM); GM-CSF + PD98059 (■ 50 µM); GM-CSF + LY294002 (□ 10 µM). Values presented are means of time 0 values (n = 4, ± S.D.).

Mcl-1 was regulated by the activity of the proteasome, and we could find no role for calpains or caspases under the experimental conditions employed in this study.

Previous published work has shown that Mcl-1 has a very short half-life, and sequence analysis has suggested that this high turnover rate is because of motifs within the protein that target it for turnover via the proteasome (11, 13, 37). The experiments described in this report directly confirm this proposal because inhibition of proteasome function resulted in a greatly decreased rate of Mcl-1 turnover. The data also show that inhibition of proteasome function can, in parallel with increasing Mcl-1 stability, significantly delay commitment to apoptosis. However, in many cell types, such as some cancer cells, inhibition of proteasome function accelerates apoptosis or sensitizes the cell to induction of apoptosis (38, 39). This would imply a role for the proteasome in regulating the activity of a death protein(s) in these cells: blocking the turnover of such a protein would allow it to accumulate and thus render the cells more sensitive to death by apoptosis. Indeed, such observations have led to the proposal that proteasome inhibitors may have clinical benefit for anti-cancer therapy (39). The data presented in this report again highlight a key role for the proteasome in regulating apoptosis in neutrophils but with a completely opposite effect to that seen in cancer cells. In neutrophils, blocking proteasome function promotes cell survival. Although we have focused on changes in turnover rates of Mcl-1 because of its key role in regulation of neutrophil cell death and survival, it will now be of interest to determine whether other proteins in neutrophils with normally short half-lives are "stabilized" by GM-CSF. Identification of such proteins would be important for defining their roles in these normally short lived cells.

Sequence analysis of Mcl-1 identifies several potential phosphorylation sites, and it has been shown that hyperphosphorylation of the protein can be detected as a band shift on SDS-PAGE and Western blotting (40). It has also been shown that in Hek292 cells, Mcl-1 can be phosphorylated on Ser121 and Thr163 via a c-Jun NH2-terminal kinase-dependent pathway in response to oxidative stress (41). In Burkitts lymphoma cells BLA1–3, Mcl-1 may be phosphorylated on two levels: phorbol esters can induce a phosphorylation via Erk that does not result in a band shift, whereas Taxol and okadaic acid can induce Mcl-1 hyperphosphorylation that does result in a band shift on SDS-PAGE (40). Based on these observations and our new data, we now propose that this differential phosphorylation of Mcl-1 results in dynamic changes in its stability.

Based on the data presented in this report, we propose that GM-CSF signals a decrease in Mcl-1 turnover via phosphorylation through Erk/Mek and PI3K/Akt signaling pathways but this phosphorylation does not result in a band shift on SDS-PAGE and Western blotting. This increased stability of Mcl-1 is sufficient to confer increased resistance to apoptosis. However, this process slows but does not stop Mcl-1 turnover, and it is noteworthy that activation of Erk and Akt following GM-CSF treatment is only transient and falls rapidly by 30 min post-stimulation. We propose that Mcl-1 may also be phosphorylated on different sites within the protein (presumably by different kinases) and that this hyperphosphorylation targets the protein for turnover via the proteasome. This turnover of Mcl-1 then triggers neutrophil apoptosis. The kinase(s) responsible for this Mcl-1 hyperphosphorylation is unknown. Based on its complex kinetics of activation of p38-MAPK and previous reports in the literature (reviewed in Ref. 1), we thought that p38-MAPK might be involved. However, experiments using the inhibitors SB202190 or SB203580 (data not shown) did not support this conclusion. However, these inhibitors only block the activities of the α and β isoforms of p38 and have no effect on the γ and δ isoforms (SAPK3 and SAPK4) (42). Unfortunately, no specific inhibitors yet exist for these latter two isoforms of p38, and so we cannot rule out the possibility of their involvement in Mcl-1 turnover.

The function of a large number of Bcl-2 family members is controlled by their phosphorylation status. For example, Bcl-2 is phosphorylated at Ser70 to enhance its anti-apoptotic activity, but its hyperphosphorylation by drugs such as Taxol at Thr69 and Ser75 within the flexible loop region may inhibit its function and enhance proteolysis via the proteasome (43–45). Bcl-XL function is also regulated via its phosphorylation status (46), whereas Akt/PI3K-dependent phosphorylation of Bad on Ser112, Ser155, and Ser159 may promote association with 14-3-3 protein within the cytoplasm, thereby releasing it from Bcl-XL and allowing Bcl-XL to heterodimerize with pro-apoptotic proteins to promote survival (47–53). Thus, phosphorylation events can exert profound effects on the cell death machinery of cells by activating, inactivating, or enhancing the turnover of anti- or pro-apoptotic proteins. It is thus likely that differential phosphorylation of Mcl-1 in neutrophils may also exert positive and negative effects on its function: phosphorylation on specific residues regulating activity and stability, phosphorylation on other residues enhancing turnover.

Although we have measured rates of Mcl-1 turnover, it is possible that GM-CSF signaling does not directly affect Mcl-1 itself but rather affects the binding and/or activity of a binding partner that results in altered Mcl-1 phosphorylation and turnover. Several Mcl-1-binding proteins have been proposed, including fortillin, DAD, and Bax (54–56). Some of these are...
known to undergo functional changes during phosphorylation; it will now be interesting to determine whether GM-CSF signaling affects the phosphorylation states or turnover rates of these proteins or their ability to bind Mcl-1. Thus, the effects of GM-CSF signaling on Mcl-1 stability may be because of its effects on the function of these Mcl-1 binding partners.

In summary, we have shown that neutrophil apoptosis is directly regulated, at least in part, by the rate of turnover of Mcl-1 and that cytokine-mediated delay of apoptosis can be explained by increased Mcl-1 stability. In view of the importance of neutrophils in inflammation and inflammatory diseases, it will now be interesting to determine whether such phenomena explain decreased neutrophil apoptosis in disease and whether other proinflammatory, anti-apoptotic cytokines can also mediate these effects. In view of the growing awareness of dysfunctional Mcl-1 expression in a variety of human diseases, it will also be important to determine whether its overexpression in malignancy is because of enhanced transcription of MCL-1 or dysfunctional turnover of the protein (57–59). Defining these phenomena will be important for the design of therapeutic strategies.

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