Cooperative Regulation of Mcl-1 by Janus Kinase/STAT and Phosphatidylinositol 3-Kinase Contribute to Granulocyte-Macrophage Colony-Stimulating Factor-Delayed Apoptosis in Human Neutrophils


*J Immunol* 2001; 166:7486-7495; doi: 10.4049/jimmunol.166.12.7486

http://www.jimmunol.org/content/166/12/7486

---

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

---

**References**

This article cites 59 articles, 30 of which you can access for free at: http://www.jimmunol.org/content/166/12/7486.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2001 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Cooperative Regulation of Mcl-1 by Janus Kinase/STAT and Phosphatidylinositol 3-Kinase Contribute to Granulocyte-Macrophage Colony-Stimulating Factor-Delayed Apoptosis in Human Neutrophils


Polymorphonuclear neutrophils (PMN) are phagocytic cells constitutively programmed for apoptotic cell death. Exposure to GM-CSF delays apoptosis as measured by annexin-V staining and cell morphological change. We found that STAT5B, STAT1, and STAT3 DNA-binding activity was induced by GM-CSF. We also detected activation of the phosphatidylinositol 3-kinase (PI 3-kinase) pathway after GM-CSF treatment which was inhibited by treatment with the PI 3-kinase inhibitors, wortmannin and LY294002. We investigated whether STAT or PI 3-kinase activity was necessary for the pro-survival response of GM-CSF in PMN. Exposure of PMN to GM-CSF in the presence of either AG-490, antisense STAT3 oligonucleotides, or wortmannin resulted in a partial inhibition of GM-CSF-mediated pro-survival activity. GM-CSF induced a time-dependent increase in the mRNA and protein expression of the anti-apoptotic Bcl-2-family protein, Mcl-1. We examined the hypothesis that Janus kinase/STAT and PI 3-kinase regulation of Mcl-1 contributed to GM-CSF-delayed apoptosis. Using either AG-490 or wortmannin alone, we observed a dose-dependent inhibition of GM-CSF-induced Mcl-1 expression. Using suboptimal doses of AG-490 and wortmannin, we found that both drugs together had an additive effect on delayed apoptosis and Mcl-1 expression. These data suggest that cooperative regulation of Mcl-1 by the Janus kinase/STAT and PI 3-kinase pathways contribute to GM-CSF-delayed apoptosis. The Journal of Immunology, 2001, 166: 7486–7495.

Homostatic mechanisms that control growth, differentiation, survival, and death are tightly regulated. Disease pathogenesis results from dysregulation of signaling that controls these critical events. Human polymorphonuclear leukocytes (PMN) are phagocytic cells that are fully differentiated in circulation and provide a unique model for the study of survival signaling. PMN are programmed for cell death by apoptosis, but the lifespan of PMN is prolonged by stimuli such as GM-CSF, IL-2, IL-1, IL-8, or bacterial products such as LPS to potentiate the inflammatory response (1–6). Due to the fully differentiated nature of PMN they do not possess the capacity to proliferate in response to these factors; therefore, enhanced survival is mediated by prevention of apoptosis (7).

Our laboratory and other investigators have identified that the GM-CSF-mediated survival pathway in PMN is linked to activation of the Src family tyrosine kinase, Lyn (2). We demonstrated that GM-CSF treatment resulted in the rapid activation of Lyn kinase activity but not Hck or Fgr. Antisense oligonucleotides to Lyn prevented the antiapoptotic activity of GM-CSF. GM-CSF elicits its response by binding to surface receptors on the cell that activate a cascade of intracellular signaling events, including both serine/threonine and tyrosine phosphorylation (8). Phospholipase A2 and phosphatidylinositol 3-kinase (PI 3-kinase) activation are important in the activation of further downstream targets such as protein kinase B/AKT and transcription factors such as cAMP response element binding protein (CREB) (9–12). In addition to Lyn tyrosine kinase, c-Fps/Fes and JAK family tyrosine kinases are also activated by GM-CSF in PMN (2, 13–16). JAK2 phosphorylation occurs in response to GM-CSF in PMN that reportedly activates the STAT family proteins STAT1, STAT3, and STAT5 (14, 17–18). Other than the involvement of Lyn tyrosine kinase, little is known about the signaling pathways involved in the survival signaling of PMN. PI 3-kinase activation has also been linked to antiapoptotic activity in PMN. The target proteins that are regulated by these signaling pathways that control cell survival are poorly understood. Interestingly, we and others have demonstrated that key antiapoptotic and cell cycle regulatory proteins such as Bcl-2, Bcl-XL, p53, cdc2, and Rb fail to be expressed in either freshly isolated or GM-CSF-treated PMN (2, 19–24). Therefore, the role of other Bcl-2 family proteins is of interest. This study has identified that Mcl-1, an antiapoptotic protein structurally related to Bcl-2, is an important downstream element in GM-CSF-mediated cell survival signaling of PMN by both the STAT and PI 3-kinase signaling pathways. We found that Mcl-1 is expressed...
constitutively in human PMN, and treatment with GM-CSF induced Mcl-1 protein expression (21, 23, 25–26). More importantly, we provide the first evidence that both the STAT3 and PI 3-kinase pathways cooperatively regulate cell survival activity of GM-CSF through Mcl-1 expression.

Materials and Methods

Cell culture and cytokine induction
Leukocyte buffy coats were obtained from normal volunteers of the Southwestern Florida Bloodbank (Tampa, FL). Buffycasts were diluted with PBS (BioWhittaker, Walkersville, MD), and PBMC were separated from PMN by centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ). The PMN layer was further purified by hypotonic lysis of contaminating red blood cells and washed with PBS. PMN were shown to be at least 95% pure by flow cytometry (data not shown). The freshly isolated PMN were treated with 1000 U/ml GM-CSF (Immunex, Seattle, WA) for the indicated time periods at 37°C and used in apoptosis assays or washed with ice-cold PBS and lysed for EMSA and Western blot analysis.

Treatment with pharmacologic inhibitors and antisense STAT3
PMN were isolated, and pharmacologic inhibitors were added. The compounds used in these experiments included AG-490 (Calbiochem, La Jolla, CA), wortmannin (New England Biolabs, Beverly, MA), and LY294002 (Calbiochem) that were added 1 h before GM-CSF treatment. PMN were cultured with GM-CSF for the indicated duration before lysis for Western blot analysis. PMN from the same donor were also placed in 24-well plates for 42 h with and without GM-CSF for analysis of apoptosis. Antisense STAT3 (5'-ACT CCA ACT GCC CTC CTG CT-3') or Ab supershifting experiments, excess cold probe (100 molar ratio) or Ab was added to the incubation reaction for 30 min at room temperature in a 10-μl reaction volume. Blocking or supershifting polyclonal rabbit Abs to STAT1 (clone C136), STAT3 (clone H190), STAT5B (pan-STAT5, clone C17), and STAT5A (clone L20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The following oligonucleotide probes and the complementary sequences were synthesized and allowed to anneal in equal molar amounts prior to labeling and use in binding assays: hSIE (5'-CTTATTCCCGATATACTCT-3') (29), MgFe (5'-AGATTTCTAGGAATTCACA-3') (30), and nonrelated DNA FIRE (5'-GTCCTCCCCTCCGCGGGAGGCCT-3') (28), all from Life Technologies (Grand Island, NY). The oligonucleotide probes (2 pmol) were labeled with [32P]dATP and dCTP and the DNA probe complexes were separated on a nondenaturing 5% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer.

Cell lysate preparation and EMSA
Nuclear and cytoplasmic lysates for EMSA were prepared as previously described, and aliquots of the supernatant were stored at −70°C (28). For use as positive controls, a similar protocol was used to isolate cytoplasmic and nuclear extracts from U266 multiple myeloma cells, PMN stimulated with 1000 U/ml IFN-γ, and SKBR-3 breast cancer cells treated with epidermal growth factor, as previously described (27). Antisense STAT3 or control oligonucleotide was added to the cells under serum-free conditions. Preincubation was continued for 6 h and then 5% FCS and GM-CSF were added. The cells were further incubated for 18 h and then lysed for Western blot analysis or prepared for apoptosis assays and cell viability staining.

RNAse protection assay (RPA)
Cell pellets were lysed in TRIzol reagent, and total RNA was isolated according to the manufacturer’s protocol (Life Technologies). RNA was quantitated at 260 nm and aliquoted at −70°C. Probes were synthesized using the Bcl-2 family, multiple-probe template set hAPO-2 (PharMingen), and 10 μg RNA per sample was prepared for electrophoresis using In Vitro Transcription Kit (PharMingen). The probes were resolved on a 5% denaturing polyacrylamide gel, dried, and autoradiographed. The image was quantitated by densitometry using ImageQuant software.

Apoptosis assay
Freshly isolated PMN in complete medium were placed in 24-well tissue culture plates at a cell density of 1×10⁶ cells/ml in 0.5 ml per well in the presence or absence of GM-CSF. Pharmacologic inhibitors were added for the time indicated in each experiment, and the cells were washed in sample wash buffer (PharMingen) and stained with either annexin-V-FITC alone (PharMingen) or in combination with PI according to manufacturer’s recommendation. In experiments where PI was used, it was added as an indicator of viability and no distinction was made between intermediate and late apoptosis. Cells that stained positively for annexin-V-FITC were considered apoptotic. Data acquisition and analysis was performed by the Flow Cytometry Core Facility at the H. Lee Moffitt Cancer Center, (Tampa, FL).

Western immunoblotting and immunoprecipitation
Freshly isolated PMN (1×10⁷) were lysed in 1 ml buffer composed of 50 mM Tris-Cl, pH 7.6, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100 containing 1 μg/ml leupeptin, aprotinin, and antipain, 1 mM sodium orthovanadate, and 0.5 mM PMFSF (all obtained from Sigma, St. Louis, MO). The total protein was estimated, and 50 μg of denatured protein was analyzed on a 10% SDS-polyacrylamide gel. Western immunoblotting was performed with the following Ab dilutions: anti-Mcl-1 (Santa Cruz Biotechnology), anti-Bax (rabbit polyclonal; PharMingen, San Diego, CA), anti-PO_AKT (clone 9271L; New England Biolabs), anti-totalAKT/PKB (catalog no. 06-558; Upstate Biotechnology, Lake Placid, NY), and anti-STAT3 (clone H190; Santa Cruz Biotechnology) at a dilution of 1:1000; anti-β-actin (Sigma) at 1/2500 dilution; and anti-JAK2 (Santa Cruz Biotechnology) at 1/200; anti-phosphoSTAT3 (clone 9131S; New England Biolabs) and anti-phosphotyrosine (clone 4G10; Upstate Biotechnology) at 1/500 dilution. The blots were then incubated with anti-rabbit or anti-mouse IgG-conjugated HRP (Amersham, Arlington Heights, IL) and visualized by ECL (Amersham). The gels were scanned by densitometry and the signal was quantitated using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation was performed by the addition of 25 μl of protein A- or protein G-Sepharose beads to cell lysates from 2.5×10⁶ PMN. The immune complexes were washed twice with 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.5% NP-40 containing 1 μg/ml leupeptin, aprotinin, antipain, and 0.5 mM PMFSF followed by one wash with 50 mM Tris-Cl, pH 7.6, 150 mM NaCl (buffer A) and 1 wash with Tris-Cl, pH 6.7 (buffer B). The complexes were removed by boiling in Laemmli SDS-PAGE sample loading buffer and analyzed on a 10% SDS-PAGE gel.
Results

**Kinetic analysis of GM-CSF-induced prevention of apoptosis**

We and other investigators have demonstrated that GM-CSF delays the apoptotic cell death of PMN (1–2, 6, 9). A hallmark of apoptosis is the appearance of phosphatidylserine in the external plasma membrane that can be detected by binding to annexin-V-FITC by flow cytometry. Although externalization of phosphatidylserine has been described in medium-cultured PMN, the kinetics has not been determined with GM-CSF treatment (31). We first examined the expression of phosphatidylserine and found that it was highly expressed in PMN undergoing apoptosis, and the binding of annexin-V-FITC was reduced by treatment with GM-CSF at 42 h (Fig. 1). This quantitative method was used to describe the level of apoptosis in further experiments and confirmed by microscopic examination.

**JAK/STAT pathway induced by GM-CSF**

The Janus kinase (JAK)-family of tyrosine kinases includes JAK1, JAK2, JAK3, and TYK2 (32–33). Of the JAK family proteins, JAK2 was shown to be activated by GM-CSF in PMN (12, 17, 18). The downstream targets for activated JAK family tyrosine kinases are members of the STAT family transcription factors (14–17). We hypothesized that JAK-STAT signaling contributed to cell survival due to the previously published results linking STAT-activation to the transcriptional regulation of antiapoptotic proteins (34). Importantly, IL-6 activation of STAT3 in multiple myeloma cells was demonstrated to protect from apoptosis by transcriptional regulation of Bcl-XL (34). Also, STAT activation is strongly associated with tumorigenesis of oncoproteins such as v-src and v-abl (28, 35, 36). We evaluated whether the STAT proteins STAT1, STAT3, and STAT5 were activated in GM-CSF-treated PMN by EMSA. Using a 32P-labeled probe that has been demonstrated to recognize both STAT5 (MGFe) and STAT1 homodimers, we found robust activation of STAT5-DNA binding activity that was competed by cold MGFe but not a nonrelated DNA sequence (FIRE) (Fig. 2a). We then performed supershift analyses of IL-2, GM-CSF, and IFN-γ-treated PMN extracts with Abs to STAT1, STAT3, STAT5B (pan-STAT5), and STAT5A (specific to STAT5A) (Fig. 2b). IL-2 and GM-CSF treatment induced STAT5-like DNA binding activity. Anti-STAT1 and anti-STAT3 Abs failed to eliminate or supershift the complex, respectively. A pan-STAT5 Ab that recognizes STAT5A and STAT5B supershifted the entire complex activated by IL-2. A partial supershift with anti-STAT5A Abs confirmed the presence of both activated STAT5A and B in the complex of IL-2-activated PMN (Fig. 2b, lanes 7–10). Extracts from GM-CSF-treated PMN were only supershifted by anti-STAT5B Ab, indicating that the DNA binding activity contained STAT5B (Fig. 2b, lane 15). STAT5B was previously shown by others to be activated by GM-CSF in neutrophils (18).

To examine whether GM-CSF induced STAT3 activation, EMSA was performed with a high-affinity STAT3 binding sequence six-inducible element (SIE) (Fig. 2c) that binds STAT3:3 homodimers, STAT1:3 heterodimers, and STAT1:1 homodimers. GM-CSF activated a complex that bound this probe, and by supershift analyses the presence of STAT3 and, to a lesser extent, STAT1 was identified. In contrast, the activity that predominated in IFN-γ-treated PMN was STAT1. These data are in agreement with those of other investigators, which demonstrated activation of STAT1 and STAT3 using Western immunoblotting with phosphorylation-specific Abs (17, 18).

**Cell viability**

PMN were treated with pharmacologic inhibitors as described for the apoptosis assay. After treatment, cells were washed in RPMI 1640 medium and resuspended at a concentration of 2 × 10⁵ cells/0.1 ml, and cytopsins were made in duplicate. The slides were stained with modified Wright-Giemsa. Cells demonstrating condensation of the nuclei were considered apoptotic; each slide was examined by two individuals with one person blinded.

**FIGURE 2.** GM-CSF-induced activation of STAT DNA binding. Freshly isolated PMN were incubated for 5 min with medium (lanes 1 and 2), GM-CSF (lanes 2, 5, and 7–9), and IFN-γ (lanes 3 and 6). SKBR-3 treated with EGF for 5 min was used as a positive control. In a and b EMSA was performed with 32P-labeled oligonucleotide probes that recognize STAT5 (MGFe) and in c STAT3 (hSIE). Antibody supershifting experiments were performed using anti-STAT1 (b, lanes 7, 13, 18, and c, lanes 3, 7, and 11), anti-STAT3 (b, lanes 8, 14, and 19, and c, lanes 4, 8, and 12), anti-STAT5a/b (b, lanes 3, 9, 15, and 20, and c, lanes 5, 9, and 13). The results shown are representative of three separate experiments.
Reversal of GM-CSF antiapoptotic activity by AG-490

The role of STAT activation in GM-CSF-mediated delay in apoptosis was evaluated using the JAK-selective tyrphostin pharmacologic inhibitor, AG-490 (37). AG-490 was demonstrated to be a selective inhibitor of both JAK2 and JAK3 kinase activity and thereby reduce STAT activation (38). Increased phosphorylation of JAK2 was detected in immunoprecipitates of GM-CSF-treated cells that was reduced with increasing doses of AG-490 pretreatment (Fig. 3a). Furthermore, we also found that STAT3 phosphorylation by GM-CSF was inhibited by AG-490 (Fig. 3b). To determine if JAK/STAT activation is related to GM-CSF-delayed apoptosis, we performed apoptosis assays using GM-CSF-treated PMN from the same donor after 42 h. Indicative of GM-CSF-mediated antiapoptotic activity, there was a reduction in the percentage of apoptotic cells with GM-CSF treatment in comparison to medium-cultured cells (Fig. 3c, p < 0.05 by χ² analysis). In contrast, PMN treated with GM-CSF and AG-490 demonstrated enhanced cell death in comparison to GM-CSF-treated PMN (Fig. 3d, p < 0.05 by χ² analysis). These data suggest that other pathways contribute to GM-CSF-induced cell survival.

Assessment of PMN apoptosis by morphologic examination

A prominent feature of PMN apoptosis is condensation of multilobular nuclei to small condensed nuclear bodies easily identified by light microscopy after cell staining (2, 5–7). To confirm that the results obtained by annexin-V-FITC binding correlated with apoptotic morphology, we performed experiments with GM-CSF

**FIGURE 3.** The JAK/STAT pathway participates in GM-CSF-mediated survival. PMN were incubated for 15 min with medium (lane 1) or GM-CSF (lanes 2–6) in the presence of drug solvent (DMSO, vehicle control) (lane 3) or increasing doses of AG-490 (12.5, 25, and 50 μM). a, Immunoprecipitation with an anti-JAK2 Ab and Western blot analysis with anti-phosphotyrosine (anti-pTyr) was used to determine the degree of JAK2 phosphorylation. The relative expression of phosphorylated JAK2 to total JAK2 was determined by densitometry and found to be 0.1 in medium-cultured PMN, 0.4 in GM-CSF-treated PMN, and relative levels of 0.5, 0.2, and 0.08 in PMN treated with GM-CSF with 12.5, 25, and 50 μM AG-490, respectively. b, Western blot analysis with a phosphorylation-specific STAT3 Ab was used to determine the amount that STAT3 was activated by GM-CSF and then total STAT3 analysis was performed to demonstrate equal loading of STAT3 protein in each lane. c, Histograms of annexin-V-FITC binding in PMN treated for 42 h with medium, GM-CSF, GM-CSF + DMSO, and GM-CSF + AG-490. Experiments were performed in duplicate and the results shown are the mean ± SD from one of three similar experiments. Percent reduction of annexin-V binding in comparison to medium-cultured cells is shown on the right. d, Percentage of PMN that bound annexin-V-FITC after medium, GM-CSF, GM-CSF + DMSO (drug vehicle control), or increasing doses of AG-490 (25, 50, and 100 μM) after 42 h of culture. The percent reduction in annexin-V binding in comparison to medium-treated PMN is shown on the bottom. e, Morphology was examined in neutrophils treated with medium (a and b) and GM-CSF (c–e) after the addition of drug vehicle control, DMSO (DM) (d), or the pharmacologic inhibitor, AG-490 (e). Experiments were performed in duplicate. The % apoptotic cells is shown on the bottom of each panel with both intermediate and late apoptotic cells included in the calculation. The total number of cells counted per experiment is also shown on the bottom of each panel. Statistical analysis using a χ² test revealed that GM-CSF significantly reduced the amount of apoptosis in PMN (p < 0.05) and that this reduction was partially reversed by the addition of AG-490 (p < 0.05) but remained statistically less than medium-cultured PMN (p < 0.05). Normal neutrophil morphology (arrow head), intermediate apoptosis (long arrow), and late apoptosis (short arrow) are indicated.
Antisense STAT3 reduced GM-CSF-mediated cell survival

By EMSA DNA-binding studies, we found that STAT1, STAT3, and STAT5B were activated in response to GM-CSF in PMN. Others have previously demonstrated similar results for STAT activation but the biological significance was not determined (14, 17, 18). An antisense oligonucleotide approach to reduce intracellular STAT3 protein expression is an effective means of dissecting the direct contribution of this protein to GM-CSF-mediated cell survival (39). The STAT3 oligonucleotide treatment in comparison to control oligonucleotide partially but not completely reduced STAT3 expression in medium and GM-CSF-cultured PMN by Western blot analysis (Fig. 4a). In parallel assays, the PMN treated with GM-CSF and antisense STAT3 displayed increased annexin-V-FITC staining (Fig. 4b) and reduced viability (Fig. 4c) in comparison to PMN cultured for 24 h with control oligonucleotide. Therefore, using a method that specifically targeted the STAT3 protein, we confirmed that STAT3 signaling participates in GM-CSF-mediated cell survival.

Induction of Mcl-1 protein and mRNA expression by GM-CSF.

Apoptosis is tightly regulated by Bcl-2 family proteins, some of which function to suppress apoptosis (Bcl-2, Bcl-xL, Mcl-1, Bfl-1/A1, and A20) and others that potentiate apoptosis (Bax, Bad, Bid, Bok, Bcl-xs) (40–48). Our laboratory and others have previously shown that PMN fail to constitutively express Bcl-2 and Bcl-xL antiapoptotic proteins (2, 20–25). In contrast, Mcl-1 protein was shown to be constitutively expressed and inducible by GM-CSF, IL-1β, sodium butyrate, and LPS (21). We first examined the level of mRNA expression of several Bcl-2-family members by RTPAs after 1, 8, and 18 h of GM-CSF treatment. Here, the antiapoptotic proteins constitutively expressed in PMN were Bfl-1/A1, Bcl-w (data not shown), and Mcl-1 (Fig. 5a). These data are in agreement with previously published results for constitutive expression of antiapoptotic proteins in PMN (21–23, 49). Bfl-1/A1 is known to be inducible by some but not all survival enhancing treatments. In our study, GM-CSF treatment resulted in the induction of both Bcl-xL and Mcl-1 mRNA but not Bfl-1/A1 or Bcl-w (Fig. 5a). We found that Bcl-2 mRNA was not expressed constitutively or induced by GM-CSF. We then examined the expression of Bcl-xL and Mcl-1 proteins by Western blot analysis. After treatment with GM-CSF, Mcl-1 protein expression was increased in comparison to β-actin (Fig. 5b); Bcl-xL protein was not detected (Fig. 5b). Continued incubation of PMN with GM-CSF for 18 h did not result in detectable Bcl-xL protein induction (data not shown).

Coimmunoprecipitation of Mcl-1 and Bax

The relative ratio and dimerization partnerships of antiapoptotic and proapoptotic Bcl-2-family proteins are critical determinants of life and death (50). Several experimental models of Mcl-1 overexpression has confirmed that this protein protects cells against apoptotic cell death (51–54). Protection against Bax-mediated cell death occurred when Mcl-1 was overexpressed in a yeast two hybrid system (55). Therefore, we evaluated whether association between Mcl-1 and Bax could be detected by coimmunoprecipitation in PMN. Using a mouse mAb to Bax for immunoprecipitation of untreated or GM-CSF (18 h)-treated PMN, we detected Mcl-1 in Bax immunoprecipitates by Western immunoblotting with a rabbit polyclonal anti-Mcl-1 Ab (Santa Cruz Biotechnology) (Fig. 6). In the presence of GM-CSF, there was a 3-fold increased amount of Mcl-1 coimmunoprecipitated with Bax as determined by densitometry. The relative rise in Mcl-1 expression and enhanced association with Bax after GM-CSF treatment supports our hypothesis that Mcl-1 plays an important role in GM-CSF-delayed apoptosis.
PI 3-kinase regulates GM-CSF-induced Mcl-1 expression and antiapoptotic activity

Induction of Mcl-1 in BaF3 cells treated with IL-3 or GM-CSF has been linked to the activation of PI 3-kinase/AKT (10, 12, 55, 56). Therefore, we examined the level of Mcl-1 expression in the presence of GM-CSF alone and in combination with PI 3-kinase inhibitors. GM-CSF induced rapid phosphorylation of AKT that was inhibited by wortmannin and LY294002 pretreatment, suggesting that the PI 3-kinase/AKT pathway was activated (Fig. 7a). We found that a dose of 100 nM wortmannin and 25 μM LY294002 completely reversed the AKT phosphorylation as determined by densitometry. Additionally, we found that increased doses of both wortmannin and LY294002 reduced the GM-CSF-mediated induction of Mcl-1 protein expression after 24 h. The relative densitometry measurements obtained in comparison to β-actin were determined with the following data obtained: medium (0.13), GM-CSF (0.42), GM-CSF with wortmannin 50 (0.34), 100 (0.23), and 200 nM (0.2), GM-CSF with LY294002 25 (0.13) and 50 μM (0.07) (Fig. 7a). We found that the expression of Bax was unchanged by any of these treatments. Activation of the PI 3-kinase/AKT pathway has often been linked to survival signaling in PMN (19). Using wortmannin in combination with GM-CSF, we observed increased apoptosis by annexin-V binding (Fig. 7b) and microscopic examination (Fig. 7c) in contrast to GM-CSF-treated PMN. Also, treatment of PMN with increasing doses of wortmannin or LY294002 progressively increased the percentage of apoptotic cells in GM-CSF-treated PMN (Fig. 7d). These data suggest that not only the STAT3 signaling pathway but also the PI 3-kinase pathway regulate GM-CSF-delayed apoptosis. One downstream effector of the PI 3-kinase pathway is the induction of antiapoptotic Mcl-1 expression.

Involvement of STAT proteins in Mcl-1 induction

The role of STAT activation in Mcl-1 expression was evaluated using AG-490. We used doses of AG-490 known to inhibit both JAK2 phosphorylation and GM-CSF-mediated apoptosis. We then evaluated the level of Mcl-1 protein after incubation with AG-490 or an equal volume of DMSO (vehicle control) (Fig. 8). There was a progressive decline in Mcl-1 protein expression in PMN cultured in medium consistent with rapid degradation of Mcl-1 previously linked to the presence of a “PEST” sequence in the protein (Fig. 8, lanes 1 and 2) (48). However, the amount of Mcl-1 protein was...
FIGURE 7. Activation of PI 3-kinase/AKT contributes to Mcl-1 inducible expression and survival signaling by GM-CSF. PMN were treated for 2 h with 50, 100, and 200 nM wortmannin or 25 and 50 µM LY294002 before addition of GM-CSF for 24 h. a, Western blot analysis was performed after 24 h with anti-phosphoAKT, anti-AKT, anti-Mcl-1, anti-Bax, and anti-β-actin. The culture was continued in these cells for 42 h with medium, GM-CSF, or GM-CSF + 100 nM wortmannin. The cells were then evaluated for annexin-V-FITC and PI binding by flow cytometry (b) or morphologic examination (c). PI was added as a viability indicator. The percentage of reduction in the number of annexin-V-FITC-positive cells in comparison to medium-cultured cells was calculated and shown at the bottom of each panel. d, Annexin-V binding assays to determine the degree of apoptosis in PMN treated with medium or GM-CSF in the presence of increasing doses of wortmannin and LY294002.
higher in GM-CSF-treated extracts at both time points with a partial reduction in Mcl-1 protein in AG-490-treated cell extracts (Fig. 8). These data are consistent with the involvement of the JAK/STAT pathway in GM-CSF-induced Mcl-1 expression in addition to that of PI 3-kinase.

Cooperation between the PI 3-kinase and JAK/STAT pathways

Using either AG-490 or wortmannin alone, we consistently observed only partial inhibition of Mcl-1 induction and only partial reversal of the cell survival advantage mediated by GM-CSF. Furthermore, the STAT and PI 3-kinase pathways regulate similar biological functions in PMN suggesting that they may participate together. Therefore, we examined whether the two pathways function cooperatively by using suboptimal doses of both AG-490 and wortmannin. Using either drug alone, we observed a partial reversal of GM-CSF-reduced apoptosis (Fig. 9, a and b). Under these conditions, morphologic examination showed a mixture of nonapoptotic PMN as well as PMN with early findings of apoptosis. In contrast, however, when both drugs were added concurrently with GM-CSF, the findings were similar to cells cultured without GM-CSF for 42 h, containing almost all apoptotic PMN (Fig. 9b). Mcl-1 protein expression was greatly reduced in the cells treated with both AG-490 and wortmannin after 24 h. In contrast, the levels of Bax and β-actin were unchanged by GM-CSF or drug treatments. These data are consistent with a cooperative role for PI 3-kinase and JAK/STAT in the regulation of Mcl-1 expression and GM-CSF survival signaling in PMN.

Discussion

PMN undergo apoptosis unless survival factors are secreted that delay cell death (1–6). GM-CSF increases the life span of neutrophils but the pathways involved have not been clearly established (2, 19–25). Because the cells are terminally differentiated and incapable of proliferation, the study of PMN survival signaling is an ideal system for dissecting antiapoptotic from proliferative intracellular processes that may occur in malignancy. The contribution of both STAT proteins and PI 3-kinase to survival and proliferation has been well established in both normal and malignant cells but their role in neutrophil biology has not been defined (11, 32–34, 56, 57).

We found that the STAT pathway is an important determinant of GM-CSF antiapoptotic signaling in PMN. Our data using the AG-490 JAK-selective pharmacological inhibitor as well as an anti-
We also demonstrated that the PI 3-kinase/AKT pathway contributed to GM-CSF-mediated delayed apoptosis in PMN. These results are similar to the effects of GM-CSF observed in BaF3 cells, in which a pathway involving PI 3-kinase activation of AKT was critical for survival (56, 57). A well-defined target of AKT phosphorylation is the Ser136 site of the proapoptotic protein Bad (47). Traditionally, phosphorylation results in the binding of Bad to the 14-3-3 protein that interrupts the association between Bad and Bcl-xL or Bcl-2. Increased amounts of Bcl-xL and Bcl-2 are then free to bind Bad and prevent its proapoptotic activity. Interestingly, others have shown Bad phosphorylation in response to GM-CSF in human neutrophils; however, Bcl-xL and Bcl-2 are not present in PMN (19). These results suggest the possibility that Bad might interact with Mcl-1. However, yeast two-hybrid analyses using Mcl-1 and Bad failed to demonstrate a viability response suggesting that Mcl-1 and Bad are incapable of dimerization. It is conceivable that biologically relevant partnerships such as Mcl-1 and Bad may only occur in some cell types and fail to be detected in yeast two-hybrid studies. Future experiments will determine the role of Bad phosphorylation and its possible association with Mcl-1 in PMN.

In our experiments, we found that GM-CSF signaling led to increased expression of the antiapoptotic protein Mcl-1. Similar to the results of yeast two-hybrid and FDC-P1 cells overexpressing Mcl-1, we were able to identify a heterodimeric complex of Mcl-1 and Bax by communoprecipitation experiments (52). These are important novel findings that provide evidence of Mcl-1/Bax heterodimerization from human cell extracts without overexpression. In contrast, communoprecipitation experiments in 32D myeloid leukemia cells failed to detect Mcl-1/Bax heterodimers by communoprecipitation even with overexpression (58). Although not studied here, GM-CSF may also contribute to accumulation of the Mcl-1 protein by stabilization of the protein. However, from the data presented here, we can conclude that PI 3-kinase/AKT-mediated induction of Mcl-1 protein expression acts as an additional mechanism to positively regulate cell survival in PMN.

Increased PMN survival after GM-CSF treatment has also been attributed to activation of mitogen-activated protein kinase (MAPK), p42/44 extracellular signal-related kinase-1 (2, 19). Based upon studies by Klein et al., no additive inhibition of apoptosis delay occurred when LY294002 and a MAPK kinase (MEK) inhibitor that blocks activation of MAPK (PD098059) were added concurrently (19). These results suggest that pathways distinct from PI 3-kinase are involved in MAPK prevention of apoptosis.

Other investigators have demonstrated that AKT phosphorylation of a transcription factor complex containing CREB was involved in the up-regulation of Mcl-1 gene expression in BaF3 cells (57). Interestingly, an unidentified STAT-like transcription factor was also involved in IL-3-mediated mcl-1 inducibility. Using heterologous studies of the murine Mcl-1 promoter, these authors showed that both an SIE-STAT-like element and a CREB site were necessary to confer complete IL-3 inducibility. The human mcl-1 promoter is yet to be cloned to confirm the results of the murine promoter studies. However, we have found that STAT3 can directly regulate the murine mcl-1 promoter in NIH3T3 cells overexpressing v-src (our unpublished observations). With the confirmed results of transcriptional regulation, these data are strong evidence for cooperation between the JAK/STAT and PI 3-kinase pathways with regard to GM-CSF-inducible Mcl-1 protein expression in this biologic setting. Additional experiments are required to identify the mechanism of STAT and PI 3-kinase cooperation. These data are also consistent with a role for Mcl-1 in GM-CSF-mediated pro-survival activity, although a direct link must still be established. Using antisense oligonucleotides to Mcl-1, Moulding et al. recently showed that Mcl-1 is essential for prevention of apoptosis during differentiation of U937 cells (59). Our data provide further insight into key intracellular events that are necessary for GM-CSF-mediated regulation of apoptosis in human neutrophils.

Acknowledgments

We thank Dr. Hong-Gang Wang for his review of the manuscript and helpful comments. We also thank Dr. James Karras (Isis Pharmaceuticals) for providing the antisense STAT3 and control oligonucleotides.

References


41. Lin, E. Y., A. Orlofsky, H. G. Wang, J. C. Reed, and M. B. Prystowsky. 1996. A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differ-


43. Kozopoulos, K. M., T. Yang, H. L. Buchan, P. Zhou, and R. W. Craig. 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence sim-

44. Heyninck, K., G. Denecker, D. DeValck, W. Fiers, and R. Beyaert. 1999. Inhibi-

45. Heyninck, K., G. Denecker, D. DeValck, W. Fiers, and R. Beyaert. 1999. Inhibi-


