Published in final edited form as:
Am J Respir Cell Mol Biol. 2011 June ; 44(6): 879–887. doi:10.1165/rcmb.2010-0101OC.

GM-CSF CAUSES A PARADOXICAL INCREASE IN THE BH3-ONLY PRO-APOPTOTIC PROTEIN BIM IN HUMAN NEUTROPHILS

Andrew S Cowburn[1], **Charlotte Summers**[1], **Benjamin J Dunmore**[2], **Neda Farahi**[1], **Cristin G Print**[2,3], **Simon J Cook**[4], and **Edwin R Chilvers**[1]

[1]Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, CB2 0QQ, UK

[2]Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK

[3]Department of Molecular Medicine and Pathology, School of Medical Sciences, University of Auckland, Park Road, Auckland, NZ

[4]Laboratory of Molecular Signalling, The Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, UK

Abstract

Neutrophil apoptosis is essential for the resolution of inflammation but delayed by several inflammatory mediators. In such terminally differentiated cells it has been uncertain whether these agents can inhibit apoptosis through transcriptional regulation of anti-death (Bcl-X_L, Mcl-1, Bcl2A1) or BH3-only (Bim, Bid, Puma) Bcl2-family proteins. We report that GM-CSF and TNFα prevent the normal time-dependent loss of Mcl-1 and Bcl2A1 in neutrophils and demonstrate that they cause a NF-κB-dependent increase in Bcl-X_L transcription/translation. Surprisingly, we show that GM-CSF and TNFα increase and/or maintain mRNA levels for the pro-apoptotic BH3-only protein Bid and that GM-CSF has a similar NF-κB-dependent effect on Bim transcription and BimEL expression. The *in-vivo* relevance of these findings was shown by the demonstration that GM-CSF is the dominant neutrophil survival factor present in lung lavage from patients with ventilator-associated pneumonia and confirmation of an increase lung neutrophil Bim mRNA. Finally GM-CSF caused mitochondrial location of Bim and a switch in phenotype to a cell that displays accelerated caspase-9-dependent apoptosis. This study demonstrates the capacity of neutrophil survival agents to induce a paradoxical increase in the pro-apoptotic proteins Bid and Bim and suggests that this may function to facilitate rapid apoptosis at the termination of the inflammatory cycle.

Address correspondence to: Dr Andrew S Cowburn, Respiratory Medicine Division, Department of Medicine, University of Cambridge School of Clinical Medicine, Level 5, Box 157, Addenbrooke's Hospital, CUHNHSFT, Hills Road, Cambridge, CB2 2QQ, UK. Tel: +44 1223 336862; Fax: +44 1223 762007; asc32@cam.ac.uk.
Authors Contribution ASC, ERC, CP designed the research, ASC performed the research, BD contributed to technical assistance to the research and analysis of data, CS harvested BALF from VAP patients and provided statistical support, SJC provided intellectual input and analysis of data, NF provided some laboratory support.

Europe PMC Funders Author Manuscripts

Keywords

Neutrophil; Apoptosis; Bim; pneumonia; NF-κB; survival factors

INTRODUCTION

Neutrophils are key effector cells of the innate immune responseproviding the first line of defence against invading micro-organisms. However, persistent recruitment, activation and aberrant survival of these cells at inflamed sites appears to be an important 'driver' of neutrophil-mediated tissue damage.(1) Neutrophils have a short circulating half life (6-8h) and die rapidly by apoptosis when aged *in-vitro*(2). This form of programmed cell death also occurs *in-vivo*.(3)There is now a substantial body of data supporting the view that neutrophil apoptosis is vital for the safe clearance of senescent neutrophils under physiological conditions.(4)(5). Importantly, several growth factors and cytokines, including GM-CSF(6), G-CSF(7), IL-8 and TNFα(8) have been postulated to impede neutrophil apoptosis thereby impairing the resolution of inflammation.

The mechanisms triggering neutrophil apoptosis are subject to debate and have been proposed to reflect oxidant-induced mitochondrial damage, a time-dependent critical decline in NF-κB activity, and changes in Bcl-2 family and cIAP protein expression(9). A number of survival factors are recognised to alter the expression, phosphorylation, activation state, cellular location and binding partners of several Bcl-2-family members in neutrophils. The Bcl-2 family are major regulators of mitochondrial-integrity and mitochondria-initiated caspase activity(10). The family consist of both pro-apoptotic and anti-apoptotic members sub-divided into three main classes defined by the homology within four conserved Bcl-2 domains (BH1-4) (10). The anti-apoptotic members (including Bcl-2, Bcl-X_L, Bcl2A1 and Mcl-1) show sequence homology through BH1-4. The pro-apoptotic members are divided into Bax and Bak-like proteins and BH3-only proteins. The former group of proteins share homology in two or three BH domains. The BH3-only members (including Bim, Bid, and Puma) share homology in the BH3-region only.

Whilst generality of the heterodimeric interactions that exist between the anti-apoptotic and pro-apoptotic Bcl-2 members has been well documented, the sequence of events leading to Bax/Bak activation remains controversial. Circulating mature human neutrophil lack Bcl-2 yet this cell expresses most other anti-apoptotic members namely Bcl-X_L, Bcl2A1 and Mcl-1 as well as the pro-apoptotic proteins Bax, Bak, Bid, Bim and Puma.(11-16)Previous reports have supported a dominant role for Bcl2A1 and Mcl-1 in regulating constitutive apoptosis in neutrophils. Hence while neutrophils from mice deficient in Bcl2A1 develop normally they exhibit enhanced rates of apoptosis when cultured *in-vitro*(17). Likewise, neutrophils from Mcl-$1^{-/-}$ mice have a higher rate of spontaneous apoptosis compared to control mice crucially however, GM-CSF still has a profound survival effect in these cells. (18) This suggests the maintenance of Mcl-1 levels is unlikely to be the sole mechanism underlying the pro-survival effect of GM-CSF in neutrophils.

Bim is considered to be one of the most important BH3-only proteins in immune cells. Indeed myeloid cells life span is substantially increased in Bim$^{-/-}$ mice, blood neutrophil

counts in these animals are increased by a factor of 2.5 (19). However, the mechanism of Bim activation is not entirely clear; early reports suggested that inactive Bim is bound to the microtubule cytoskeleton and once activated translocates to the mitochondria to initiate apoptosis(20), it is now apparent that Bim is regulated at multiple levels. Hence, certain death stimuli can up-regulate Bim transcription(21) phosphorylation(22), ubiquitylation and proteasome-dependent degradation(23). The mechanism by which Bim regulates apoptosis is also uncertain as some experimental data suggest that Bim can be up-regulated without inducing apoptosis,(24) it might be required but not sufficient for cell death.

The current study's aim was to comprehensively characterise the effects of aging on the expression of the Bcl-2-family in human neutrophils determining the consequences of cytokine and growth factor-mediated survival signalling on these proteins. We wished to explore mechanisms aside from Mcl-1 to explain the survival effects of GM-CSF and to reference such effects to inflammatory neutrophils *in-vivo*. We report that GM-CSF and TNFα cause a profound and agonist-selective alteration in the balance of pro-and anti-apoptotic Bcl-2 family proteins in neutrophils, in particular maintaining the expression of Bcl-X_L and enhancing the stability of Mcl-1, but also increasing and maintaining the expression of certain pro-apoptotic proteins including Bim and Bid. Bim expression is increased in inflammatory neutrophils recovered from the lungs of patients with ventilator-associated pneumonia (VAP) where GM-CSF operates as an important survival factor. Moreover, *in-vitro* GM-CSF appears to prime neutrophils for TNFα-mediated killing. We speculate the latter effect may enable a rapid switch from survival-prone to apoptotic-prone phenotype when required thus aiding the timely resolution of inflammation.

MATERIALS AND METHODS

Isolation of human neutrophils

Human peripheral blood neutrophils were obtained from healthy non-medicated adult donors as previously detailed (see supplementary methods)(25). All human studies were approved by the local Research Ethics committee (06/Q0108/281; 08/H0306/17).. Neutrophils were cultured as previously detailed(6) in the presence/absence of GM-CSF (10ng/ml) or TNFα (10ng/ml) with/without prior incubation (30min) with the NF-κB inhibitor BAY11-7082 (10μM), PI3-kinase inhibitor LY294002 (10μM), MEK inhibitor U0126 (10μM) or JNK inhibitor SP600125 (20μM).

To assess the susceptibility of GM-CSF and G-CSF primed neutrophils to subsequent TNFα-mediated apoptosis cells were cultured ± GM-CSF (10ng/ml) or G-CSF (10ng/ml) for 6h prior to incubation with TNFα (10ng/ml) for an additional 12h. Where used, the pan-caspase inhibitor z-VAD-fmk (30μM), caspase-8 inhibitor IETD-CHO (3μM) or caspase-9 inhibitor LEHD-CHO (3μM), were added as detailed 30min before TNFα.

Assessment of neutrophil apoptosis

Neutrophils were harvested at the time points indicated, cyto-centrifuged, fixed in methanol, stained with May-Grünwald-Giemsa (Merck Ltd. Nottingham UK), and analysed as previously described(6) with the observer blinded as to the experimental variables.

Apoptosis was also assessed by flow cytometry using (i) FITC-labelled recombinant human Annexin-V (AnV)/propidium iodide (PI) staining(6) and (ii) the fluorescent cationic dye JC-1.

Real-time PCR

Total RNA (1μg) isolated with TRI-reagent (Sigma) was used for cDNA generation(high capacity cDNA kit, Applied Biosystems). Relative gene expression determined by qPCR (iCycler, Bio-Rad) using Sybr-green master-mix (Sigma) and relevant primers from Qiagen. Relative gene expression was determined by correcting cycle threshold for the target gene against five house-keeping genes (B2M, GAPDH, β-actin, YWHAZ, UBC) using genNORM (http://medgen.ugent.be/~jvdesomp/genorm) Relative gene expression (fold change) is expressed as $2^{-\Delta\Delta CT}$.

Immunoblotting

Neutrophils were lysed at the time points indicated (0, 4, 8 or 12h) in an hypotonic buffer as previously detailed(6) Lysates were separated on 15% SDS-PAGE and electrotransferred to PVDF membranes. The membranes were incubated overnight in PBS-Tw20-0.25% dried milk powder with polyclonal antibodies to Bcl-X_L, Mcl-1 (Santa Cruz), Bim (Chemicon International), Bid, PUMA, Bak (Cell signalling) or Bax (BD Pharmingen). Membranes were washed in PBS-Tw20 then incubated with peroxidase-conjugated secondary antibody (1:5000) in PBS-Tw20-0.25% dried milk powder for 1h. Detection was performed by chemiluminescence using an ECL-kit (Amersham Life Science).

Confocal microscopy

Neutrophils were pre-incubated with MitoTracker (Molecular Probes, Invitrogen) 30min before adherence to poly-l-lysine coated cover slips (15min, RT). Cells were fixed (3.7% paraformaldehyde, 20min, RT) then washed(HBSS 2×5min) and permeabilized (RPMI1640 containing 10% goat sera, 0.1% TX-100, 10mM glycine and 10mM HEPES (‘PS solution’), 30min at RT) prior to immune-staining. Coverslips were incubated at 4 °C overnight in PS solution with anti-Bim (1:250; Cell Signalling) or non-immune IgG control. Coverslips were washed with PS solution and incubated with Alexa fluor 488 goat anti-rabbit IgG diluted in PS solution (1:200, 60min, RT). Coverslips were mounted onto glass slides with Mowiol and DAPI. Cells were photographed using ultraviolet confocal microscope (TCS Leica) and images captured using ImagePro Plus 4.1. The images shown in figure-7 are composite photomicrographs created by stacking 10 images taken through the z-plan.

Isolation of peripheral blood and bronchial alveolar lavage (BALF) neutrophils from patients with VAP

BALF (0.9% NaCl) was obtained from patients with VAP, placed on ice and strained through sterile gauze to remove mucus. The BALF suspension was centrifuged (310 *g*, 5min, 4 °C) and the cells re-suspended at 50×10^6/ml in RoboSep® buffer (StemCell Technologies, Vancouver, Canada). Peripheral venous blood was drawn simultaneously into sodium citrate containing tubes; red cell sedimentation was achieved using 6% Hetastarch,

the upper layer centrifuged (310*g*, 5min, 4 °C). The supernatants were removed and stored at −80 °C, the cell pellet washed and re-suspended in RoboSep® buffer at 50×10^6 cells/ml.

The RoboSep® was used according to manufacturer's instructions and allowed simultaneous isolation of neutrophils from blood and BALF samples. This protocol generated peripheral blood neutrophils that were >99% pure and viable and BALF neutrophils >97% pure and >99% viable. Both cell suspensions were centrifuged (310*g*, 5min, 4 °C) and the pellets processed for total RNA. Neutrophils from healthy volunteers were processed in an identical manner to provide references for the qPCR data. This isolation procedure takes approximately 90 min compared to the 120 min for the standard method.

BALF immuno-deletion apoptosis assay

The BALF samples were pre-incubated with anti-G-CSF 2 μg/ml or GM-CSF 2 μg/ml antibody for 30min at RT, then diluted 1:1(v/v) with complete IMDM media containing 10^7 neutrophils/ml (final in well 5×10^6) for 18h and apoptosis assessed using Annexin-V-FITC binding (Detailed methods in supplementary section).

Measurement of BALF inflammatory mediators

BALF and serum concentrations of cytokines and growth factors were measured using commercially available ELISA kits(pro-inflammatory 9-plex human cytokine assay kit Meso Scale Discovery, Maryland, Duo-ELISA kit R&D Systems, Abingdon, UK). These data were corrected to μg total protein present in the BALF or serum sample. All samples were analysed for endotoxin contamination (Charles River, Endosafe®-IPT); all data points derived from BALF samples which had an EU reading below 0.2/ml protein.

Statistical analysis

All data represent the mean (±SEM) of (*n*) independent experiments unless otherwise stated. Differences between groups were assessed using one-way analysis of variance (ANOVA) and post hoc analysis with Tukey's multiple comparisons. A p value of <0.05 was considered significant.

RESULTS

Effects of GM-CSF and TNFα on neutrophil survival

Assessment of apoptosis using An-V-FITC demonstrated that GM-CSF reduces the rate of spontaneous apoptosis in neutrophils from 61.0±6.0% to 35.5±1.8% ($p<0.01$) at 18h (Figure 1A). TNFα also induced neutrophil survival with 46.6±3.9% ($p<0.05$) apoptosis at this time point. Representative flow cytometry data from neutrophils incubated with GM-CSF or TNFα for 18h are shown in Figure 1B. Assessment of mitochondrial membrane depolarisation using JC-1 produced near identical data with GM-CSF and TNFα both able to preserve mitochondrial membrane potential compared to the time matched controls (Figure 1A). Morphological quantification of apoptotic and non-apoptotic neutrophils confirmed the survival responses to GM-CSF and TNFα defined by An-V-FITC and JC-1 staining (Figure 1C).

Europe PMC Funders Author Manuscripts

Transcriptional and translational regulation of the Bcl-2 family in human neutrophils

Using this system we conducted comprehensive investigations of the transcriptional regulation of Bcl-2-family member genes in aged and survival factor-stimulated human neutrophils. The quality of total RNA was assessed throughout using NanoDrop (Thermo Fisher Scientific Ltd., Northumberland, UK) and separating the samples by agarose gel electrophoresis to check for degradation (see supplemental data). We investigated transcriptional regulation of eight members of the Bcl-2-family, the anti-apoptotic factors Mcl-1, Bcl-X_L, Bcl2A1 , the BH3-only members Bid, Bim and Puma (Figure 2A), and the pro-apoptotic members Bax and Bak (Supplementary Figure E1A).

The anti-apoptotic factor Mcl-1 has previously been studied and proposed to be a major regulator of neutrophil survival(26). Our data show that mRNA levels for Mcl-1 diminish markedly over time, reducing 10-fold by 12h compared to freshly isolated cells. GM-CSF and TNFα caused a minor reduction in Mcl-1 message at 2h but did not modify the subsequent time-dependent loss of mRNA. This contrasts to the ability of GM-CSF to stabilize Mcl-1 protein and prevent its degradation (Figure 2B. Supplementary Figure E2). The presence of Bcl-X_L mRNA and protein in human neutrophils has been controversial(12). Our data indicate abundant, relatively stable levels of Bcl-X_L mRNA (Figure 2A) and the ability of GM-CSF, but not TNFα, to increase Bcl-X_L mRNA expression beyond 6h ($p<0.01$) (Figure 2A). Both survival factors increased the amount of Bcl-X_L protein compared to time matched controls at 4-12h ($p<0.01$, $n=3$) (Figure 2B, Supplementary Figure E2). Bcl2A1 mRNA levels declined in a time-dependent fashion (e.g. by 49-fold at 18h) and GM-CSF and TNFα were both highly effective at preventing this decline (Figure 2A). We have been unable to detect Bcl2A1 at protein level using the currently available antibodies.

There is a consensus view that the BH3-only family are central to promoting mitochondrial-dependent apoptosis in the neutrophil. We examined Bid, Bim and Puma, known to be expressed in neutrophils.

We show that while levels of Bid and Bim mRNA both decline over time, GM-CSF caused a seemingly paradoxical rapid increase in mRNA abundance of both of these BH3-only members, which was maintained to 18h (Figure 2A), with TNFα increasing Bid but not Bim mRNA expression (Figure 2A). Previous reports investigating the expression of Bid protein in the neutrophil have described a prominent 22kDa band on Western blotting, which diminishes with caspase-dependent apoptosis, resulting in a 15kDa Bid fragment (27, 28). Bid protein (22kDa) was clearly present in freshly isolated neutrophils, diminishing rapidly over time with the appearance of a corresponding 15kDa band; in contrast, in the presence of GM-CSF or TNFα Bid expression was maintained throughout the time course (Figure 2B, Supplementary Figure E3). Of the three major isoforms of Bim (BimEL 23kDa, BimL 15kDa and BimS 12kDa) only the former two splice-variants were detected in neutrophil lysates (Figure 2B). BimL and BimEL increased over time with further increases observed in the presence of GM-CSF or TNFα at 8h (both $p<0.05$) (Figure 2B, Supplementary Figure E3). mRNA levels for Puma were stable and unaffected by GM-CSF or TNFα (Figure 2A). We could detect no consistent differences in Puma expression either over time or with GM-CSF or TNFα (Figure 2B, Supplementary Figure E3).

The mRNA expression profile for Bax and Bak was stable throughout the time course examined and only marginally affected by GM-CSF and TNFα at very late times (Supplementary Figure E1). The protein expression of Bax and Bak was also very stable and relatively unaffected by GM-CSF or TNFα (Supplementary Figure E1B, E2). These findings support the view that the major time-dependent loss in Mcl-1 and Bcl2A1 described above does not reflect non-specific loss of mRNA occurring as a consequence of constitutive apoptosis.

cIAP-1, cIAP-2, XIAP and survivin are all expressed in the neutrophil and recognised to block caspase-dependent apoptosis(29). We investigated the expression of IAP mRNAs and protein over the same time course as above (supplementary data, Figure E1A).

Neutrophil isolation from peripheral blood and BALF from patients with VAP

We considered that the most interesting aspect of the above observations was the ability of GM-CSF to cause a seemingly paradoxical increase in Bim mRNA abundance and BimEL protein expression in neutrophils *in-vitro.* Thus we wished to define whether Bim expression could be observed in inflammatory neutrophils *in-vivo* under conditions where GM-CSF may be responsible for aberrant neutrophil survival and to explore the potential physiological importance of this effect. We sought to examine Bim expression in highly pure lung neutrophils derived from patients with VAP, which we hypothesised would display GM-CSF and G-CSF-dependent neutrophil survival.

This is the first description of the successful isolation of high purity BALF neutrophils. The initial BALF samples (n=8) contained 50-70% neutrophils, this was enhanced to >98% purity using the RoboSep® isolation protocol (Figure 3A). An identical protocol was followed to isolate neutrophils from the peripheral blood from these patients and from healthy volunteers to compare gene expression.

Determination of inflammatory mediators in the BALF of patients with VAP

We measured the expression of pro-inflammatory mediators present in BALF from VAP patients and age-matched controls undergoing bronchoscopy for investigation of cough, which showed significantly elevated levels of GM-CSF (0.037pg/ml/μg protein, $p<0.05$), G-CSF (0.40pg/ml/μg protein, $p<0.05$), IL-6 (0.68pg/ml/μg protein, $p<0.005$), IL-8 (7.19pg/ml/μg protein, $p<0.005$) and TNFα (0.21pg/ml/μg protein, $p<0.005$) in the BALF from VAP compared to controls (Figure 3B). The mean values for control levels for IL-6 and IL-8 were 0.030pg/ml/μg protein and 0.55pg/ml/μg protein respectively. GM-CSF, G-CSF, and TNFα were undetectable in BALF from control subjects.

BALF neutrophils from patients with VAP express high levels of Bim mRNA

We analysed mRNA expression of the Bcl-2-family members of interest in neutrophils isolated from BALF and peripheral blood of VAP patients and compared these to healthy control cells. Bim mRNA expression was significantly higher in neutrophils isolated from the blood of patients with VAP compared to healthy subjects with a further increase in Bim expression observed in the inflammatory BALF neutrophils (Figure 3C). Additionally, Bcl-X_L mRNA was also increased in a similar pattern by 2.3-fold and 13-fold in blood and

BALF respectively when compared to healthy controls (Figure 3C). Of interest, Mcl-1 and PUMA mRNA expression was significantly lower in VAP patient neutrophil samples compared to healthy control samples (Figure 3C). The remaining Bcl-2-family members (Bax, Bak, Bid, Bcl2A1) were unchanged when compared to healthy controls (Figure 3, Supplementary Figure E4). c-IAP2 mRNA expression was also higher (8.3-fold) in the BALF neutrophils from VAP patients compared to controls although no changes were seen in c-IAP1, XIAP or survivin expression compared to healthy controls (Supplementary Figure E4).

Effect of immuno-depletion of GM-CSF and G-CSF in BALF on in-vitro neutrophil apoptosis

Previous reports have identified a potential dual role for BALF G-CSF and GM-CSF in delaying neutrophil apoptosis(7). We employed a similar approach to determine the relative importance of these growth factors in the survival effect of BALF from VAP patients using isolated neutrophils *in-vitro*. The concentration and specificity of the neutralisation antibody were pre-determined using a chequer board analysis incorporating GM-CSF (10ng/ml), G-CSF (10ng/ml) and varying concentrations of neutralising or isotype-matched control antibodies (Figure 4A, Supplementary Figure E5). Following control or active immuno-depletion of GM-CSF and/or G-CSF the BALF supernatants from 8 VAP patients were incubated with healthy donor neutrophils for 18h and the percentage of apoptosis determined. Supplementary figure E6 depicts the individual patient data and figure 4 dipicts the combined patient data. Anti-GM-CSF reversed the neutrophil survival effect of the BALF by 48.4% (range 20-75%, $p<0.005$), anti-G-CSF by 49.4% (range 32.5-68.3%, $p<0.005$) and combination by 84% (range 62.9-98.9%, $p<0.005$) (Figure 4B, Supplementary Figure E5). These percentage values were calculated using the apoptosis rates measured in the absence of added BALF (Figure 4).

These data indicate that BALF from patients with VAP contain significant amounts of active GM-CSF and G-CSF, which contribute towards the survival effect of this BALF on neutrophils and may be responsible for the elevated levels of Bim mRNA observed in the inflammatory BALF neutrophils. However, given that the expression of Bim, $BclX_L$ and Bcl2A1 is significantly higher in GM-CSF stimulated cells when compared to G-CSF stimulated cells ($p<0.05$, $n=3$, Figure 5A) we predicted that GM-CSF was the main driver of elevated Bim levels in the inflammatory BALF neutrophils and that neutrophils exposed to GM-CSF would undergo accelerated cell death following subsequent exposure to an independent death ligand.

GM-CSF primes neutrophils for TNFα-mediated apoptosis

To test the hypothesis that inflammatory neutrophils are ‘primed to die’ secondary to GM-CSF mediated increases in Bim expression, healthy cells were incubated with GM-CSF for 6h (Figure 2) followed by TNFα incubation for 12h. Under these conditions TNFα reverted from a pro-survival agent to one capable of inducing apoptosis, which was confirmed to be caspase-dependent. This effect was not seen with G-CSF (Figure 5A). Further evidence supporting the establishment of a new Bim-dependent apoptosis pathway in GM-CSF treated neutrophils was obtained using carefully titrated concentrations of caspase-8 and

caspase-9 inhibitors, which identified TNFα stimulated apoptosis to be predominantly caspase-9 (mitonchondrial) dependent in GM-CSF primed neutrophils, in comparison to a predominantly caspase-8 dependent mechanism in TNFα alone stimulated cells (Figure 5C) (7, 30).

Role of PI3-kinase, MAPK and NF-κB in the transcriptional regulation of Bcl-2-like genes

Neutrophil apoptosis is controlled by a complex network of signalling pathways. We have previously published data highlighting the importance of the PI3-kinase, MAPK and NF-κB signalling pathways in GM-CSF and TNFα stimulated survival(8). Therefore we used previously optimised concentrations of inhibitors of these pathways to dissect their importance in the transcriptional regulation of the Bcl-2-family. The expression of Bcl-X_L has been previously shown to be regulated by NF-κB.(31) Inhibition of this signalling pathway using BAY 11-7082 confirmed this finding in neutrophils (supplementary figure E7). However, this is the first report to describe the role of NF-κB in regulating the transcription of BH3-only Bcl-2-family members. We report that inhibition of NF-κB signalling reduced the basal expression of Bid in aged neutrophils by 4.3-fold at 6h ($p<0.05$) (supplementary figure E7) and that the TNFα-stimulated increase in the expression of Bid is NF-κB-dependent ($p<0.005$) (supplementary figure E7). The increase in Bim mRNA expression we observe following GM-CSF stimulation, which is the focus of this paper, was also significantly attenuated by inhibiting NF-κB signalling (supplementary figure E7). Puma and Mcl-1 expression were unaffected by NF-κB inhibition (supplementary figure E7).

Activation of the PI3-kinase/AKT pathway has been shown to repress Bim expression through phosphorylation of the transcription factor FOXO, removing the factor from its consensus binding site(32). We predicted and observed that inhibition of the PI3-kinase pathway would augment Bim expression above that seen with GM-CSF stimulation alone. We observed a 4-fold increase in Bim mRNA in cells pre-incubated with LY294002 and stimulated with GM-CSF compared with GM-CSF alone (supplementary figure E8). Inhibition of the PI3-kinase pathway did not alter the basal expression, or survival factor modulated expression of Mcl-1, Bcl-X_L, Bcl2A1, Bax, Bak, Bid or Puma (supplementary figure E8).

The MAPK inhibitor U0126 and JNK inhibitor SP600125 had no effect on the time-dependent or survival factor mediated changes in mRNA expression of any of the Bcl-2 family genes under study (supplementary figure E9).

Fluorescent distribution of Bim

Finally, confocal microscopy demonstrated diffuse and largely cytoplasmic staining of Bim in control neutrophils and only minimal co-localization with Mitotracker (Figure 6). In contrast, GM-CSF stimulation produced pyknotic Bim staining with a large degree of co-localization with Mitotracker, which was more evident following the addition of TNFα. These data suggest that GM-CSF initiates the redistribution of Bim from the cytosol to the mitochondria in addition to its effects on Bim expression.

Europe PMC Funders Author Manuscripts

DISCUSSION

Apoptosis has been proposed as one of the cardinal determinants of whether neutrophilic inflammation resolves or persists. Hence defining the mechanisms whereby this process is impeded by inflammatory cytokines or growth factors is essential. Although a number of studies have examined the presence or absence of individual components of the Bcl-2 family in human neutrophils, to our knowledge this is the first comprehensive study of the transcriptional and translational regulation of pro-apoptotic and anti-apoptotic Bcl-2-family proteins following inflammatory cytokine and growth factor stimulation. We provide evidence that the survival factors GM-CSF and TNFα induce a major shift in the balance of Bcl-2-family members at both mRNA and protein level, increasing the relative abundance of anti-apoptotic Bcl-2-members (principally Mcl-1, Bcl-X_L and potentially Bcl2A1), which appear to counteract, at least initially, the observed increase in the expression of the pro-apoptotic BH3-only members.

Interestingly, we found that GM-CSF and TNFα reduce spontaneous apoptosis in neutrophils whilst increasing the expression of Bim (GM-CSF) and Bid (GM-CSF, TNFα). Thus, in the presence of such survival agonists levels of Bim and Bid were *inversely* correlated with the overall proportion of neutrophils undergoing apoptosis. We speculate that the enhanced expression of BclX_L (GM-CSF), Bcl2A1 (GM-CSF, TNFα) and the maintenance of Mcl-1 protein expression (GM-CSF, TNFα) is able to compensate and dominate at early time points in setting the overall apoptotic threshold of the neutrophil. It appears that enhanced expression of Bim and Bid can be tolerated in these cells if matched by enhanced expression of anti-apoptotic Bcl-2 members. This conclusion is supported by recent data(33). We believe that these studies are particularly instructive as they map the outcome of physiologically relevant and fully integrated changes in Bcl-2 expression in cells lacking Bcl-2. Moreover, our studies in inflammatory neutrophils extracted from the lungs of patients with VAP indicate that GM-CSF is a major pro-survival agent in this context and that up-regulation of Bim is also observed *in-vivo*.Pre-treatment of neutrophils with GM-CSF *in-vitro* generates a cell that has acquired susceptibility to TNFα-mediated killing through a mitochondrial and caspase-9-dependent pathway. We propose that this reveals the true physiological function underlying the up-regulation of BH3-only proteins, namely to accelerate neutrophil clearance once the initial beneficial function(s) of these cells are complete.

The concept of increased expression of pro-apoptotic Bcl-2-related proteins in priming neutrophils leading to more rapid apoptosis when inflammation resolves may have precedents in other systems, e.g in the testes it is thought that male germ cells may be primed to undergo a wave of apoptosis during the first cycle of spermatogenesis by *de novo* expression of Bax and Bad in the period immediately leading up to this event(34).

The expression profile of the anti-apoptotic Bcl-2-family member Mcl-1 in myeloid cells has been well documented(35). Mcl-1 has a short half-life and is rapidly degraded by the ubiquitin-proteasome pathway in response to cytokine deprivation and other death stimuli. Agents that delay apoptosis have been shown to enhance the stability of Mcl-1 via signaling mechanisms including PI3-kinase, GSK and ERK.(26, 35-37) Our study supports the

observation that GM-CSF maintains Mcl-1 levels within the neutrophil. Additionally we show an initial increase in Mcl-1 protein levels with TNFα incubation followed by an identical rate of loss to that observed under control conditions. These data counter the view that the early pro-apoptotic effect of TNFα on neutrophils is due to a caspase-dependent cleavage of Mcl-1(38). We also report a rapid loss of Mcl-1 mRNA in aged neutrophils, which is not stabilized by GM-CSF or TNFα stimulation. While there is little doubt that Mcl-1 plays an important role in controlling apoptotic thresholds in the neutrophil(18) it is important to recognize that GM-CSF still acts as a powerful survival signal in Mcl-1 deficient neutrophils suggesting that GM-CSF can utilise alternative routes to prevent neutrophil apoptosis as shown in our study.

Neutrophils also express Bcl-X_L and Bcl2A1, two key anti-apoptotic Bcl-2-members. Enhanced neutrophil survival in septic rats has been associated with increased expression of Bcl-X_L occurring via a C5a-dependent mechanism linked to PI3-kinase and MAPK signaling(14, 15). Likewise, the enhanced numbers of neutrophils seen in the pleural cavity in a carrageenan-induced animal model of pleurisy has been attributed to increased expression of Bcl-X_L(39). These reports suggest that Bcl-X_L may also regulate neutrophil survival in many inflammatory conditions. We report that Bcl-X_L is subject to considerable transcriptional regulation, with GM-CSF able to increase and maintain high Bcl-X_L expression. Our data obtained for Bcl2A1 encompass mRNA expression only but show a surprising degree of transcriptional regulation. Hence GM-CSF and TNFα enhanced and maintained levels of mRNA for Bcl2A1, compared to rapidly diminishing levels within control cells. Together these data support the involvement of three members of the anti-apoptotic Bcl-2-family, Mcl-1, Bcl-X_L and Bcl2A1 in promoting neutrophil survival in an inflamed environment.

Bim is one of the most effective BH3-only proteins for promoting cell death and analysis of Bim deficient mice shows that Bim is a key determinant of the life span of neutrophils. We report a marked increase in the expression of Bim mRNA in neutrophils in response to GM-CSF followed by significant increases in BimEL and BimL. The levels of Bim are thus *inversely* correlated with the propensity of these cells to undergo apoptosis. These data follow closely the findings of Bauer and colleagues(40), who showed that LPS stimulation of mouse bone marrow neutrophils reduced spontaneous apoptosis, but at the same time caused a clear increase in Bim expression. We speculate that the increased expression of Bim is countered by the increased expression of Bcl-X_L, Bcl2A1 and the stabilization of Mcl-1. This supports the view offered by Andina et al that neutrophil hamatopoietins initiate a pro-apoptotic counter-regulation(33). It is therefore possible that neutrophils can tolerate the increase in Bim expression because of the corresponding increase in anti-apoptotic Bcl-2-members. The increase in Bim expression we report in neutrophils following stimulation with GM-CSF differs markedly to that seen in fibroblasts and epithelial cells where Bim is expressed *de novo* following the withdrawal of survival factors(22). Increases in Bim mRNA have been shown to result from inactivation of the Akt pathway leading to the activation of FOXO transcription factors(32) or by increased stabilization of the most abundant BimEL isoform following inactivation of the ERK1/2 pathway. Intriguingly, inhibition of PI3-kinase/Akt signaling augmented GM-CSF stimulation of Bim mRNA

levels. This indicates that while the Akt/FOXO pathway operates in neutrophils GM-CSF uses other pathways to induce Bim. Our results suggest for the first time that activation of NF-κB represents one such alternative pathway.

Bid is unique amongst BH3-only members in its ability to link the extrinsic caspase-dependent death pathway with the intrinsic mitochondrial-dependent pathway through a caspase-dependent cleavage of Bid generating truncated Bid (tBid) (41). tBid has been shown to translocate to the mitochondria to induce the oligomerisation of Bax and Bak and ultimately the release of cytochrome C(42). We report here that Bid is also under significant transcriptional regulation and that GM-CSF and TNFα increase the expression of Bid maintaining its expression as control levels diminish with time. Inhibition of the NF-κB pathway reversed this effect. Very few data exist regarding NF-κB transcriptional regulation of Bid. Protein analysis showed maintenance of Bid expression following GM-CSF and TNFα stimulation compared to rapidly diminishing values under control conditions. We were unable to detect tBid in neutrophil extracts; possibly due to the rapid turnover, protein instability or low abundance in our samples. We are aware that the diminishing amount of Bid under control condition may represent caspase-dependent cleavage of Bid during spontaneous apoptosis. However, the stabilization of Bid expression, may be countered by a corresponding increase in expression of anti-apoptotic Bcl-2-members, enabling the neutrophil to tolerate otherwise increased levels of activator BH3-only proteins. In conclusion, our results suggest the involvement of at least three members of the anti-apoptotic Bcl-2-family, Mcl-1, Bcl-X_L and Bcl2A1 in GM-CSF and TNFα-mediated neutrophil survival. This occurs in the context of a seemingly paradoxical increase in Bid and Bim. The abundance of Bim appears to be finely tuned both at a transcriptional and post-translational modification level and may determine the cellular localization and activation state of the different Bim isoforms. The precise mechanism responsible for increasing Bim and Bid expression following GM-CSF stimulation remains uncertain, but our data suggest a prominent role for NF-κB activation. Up-regulation of Bim was also observed in inflammatory neutrophils exposed to GM-CSF *in-vivo*; this increase may serve to prepare the cell for rapid apoptosis and removal at the termination of the inflammatory cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by Asthma-UK, MRC and the Cambridge NIHR Biomedical Research Centre. SJC is supported by the Babraham Institute and the BBSRC (BB/E02162X/1). The authors are grateful to Dr Doris Rassl for her help with the immunofluorescence work and Keith Burling (NIHR Cambridge BRC, Core Biochemical Assay Laboratory) for his help running the multiplex cytokine array.

REFERENCE

1. Jeffery PK. Remodeling and Inflammation of Bronchi in Asthma and Chronic Obstructive Pulmonary Disease. Proc Am Thorac Soc. 2004; 1:176–183. [PubMed: 16113432]

2. Lee A, Whyte MK, Haslett C. Inhibition of Apoptosis and Prolongation of Neutrophil Functional Longevity by Inflammatory Mediators. J Leukoc Biol. 1993; 54:283–288. [PubMed: 8409750]
3. Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. A Transgenic Zebrafish Model of Neutrophilic Inflammation. Blood. 2006; 108:3976–3978. [PubMed: 16926288]
4. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines Acting Via CXCR2 and CXCR4 Control the Release of Neutrophils From the Bone Marrow and Their Return Following Senescence. Immunity. 2003; 19:583–593. [PubMed: 14563322]
5. Perl M, Lomas-Neira J, Chung CS, Ayala A. Acute Lung Injury: Neutrophils, Epithelial Cell Death & Inflammation: A Unifying Hypothesis. Mol Med. 2008
6. Cowburn AS, Cadwallader KA, Reed BJ, Farahi N, Chilvers ER. Role of PI3-Kinase-Dependent Bad Phosporylation and Altered Transcription in Cytokine-Mediated Neutrophil Survival. Blood. 2002; 100:2607–2616. [PubMed: 12239175]
7. Matute-Bello G, Liles WC, Radella F, Steinberg KP, Ruzinski JT, Hudson LD, Martin TR. Modulation of Neutrophil Apoptosis by Granulocyte Colony-Stimulating Factor and Granulocyte/Macrophage Colony-Stimulating Factor During the Course of Acute Respiratory Distress Syndrome. Crit Care Med. 2000; 28:1–7. [PubMed: 10667491]
8. Cowburn AS, Deighton J, Walmsley SR, Chilvers ER. The Survival Effect of TNF-Alpha in Human Neutrophils Is Mediated Via NF-Kappa B-Dependent IL-8 Release. Eur J Immunol. 2004; 34:1733–1743. [PubMed: 15162444]
9. Luo HR, Loison F. Constitutive Neutrophil Apoptosis: Mechanisms and Regulation. Am J Hematol. 2008; 83:288–295. [PubMed: 17924549]
10. Cory S, Adams JM. The Bcl2 Family: Regulators of the Cellular Life-or-Death Switch. Nat Rev Cancer. 2002; 2:647–656. [PubMed: 12209154]
11. Kobayashi SD, Voyich JM, Whitney AR, DeLeo FR. Spontaneous Neutrophil Apoptosis and Regulation of Cell Survival by Granulocyte Macrophage-Colony Stimulating Factor. J Leukoc Biol. 2005; 78:1408–1418. [PubMed: 16204629]
12. Moulding DA, Akgul C, Derouet M, White MR, Edwards SW. BCL-2 Family Expression in Human Neutrophils During Delayed and Accelerated Apoptosis. J Leukoc Biol. 2001; 70:783–792. [PubMed: 11698499]
13. Ge Y, Yoshiie K, Kuribayashi F, Lin M, Rikihisa Y. Anaplasma Phagocytophilum Inhibits Human Neutrophil Apoptosis Via Upregulation of Bfl-1, Maintenance of Mitochondrial Membrane Potential and Prevention of Caspase 3 Activation. Cell Microbiol. 2005; 7:29–38. [PubMed: 15617521]
14. Guo RF, Sun L, Gao H, Shi KX, Rittirsch D, Sarma VJ, Zetoune FS, Ward PA. In Vivo Regulation of Neutrophil Apoptosis by C5a During Sepsis. J Leukoc Biol. 2006; 80:1575–1583. [PubMed: 16997861]
15. Sano J, Oguma K, Kano R, Yazawa M, Tsujimoto H, Hasegawa A. High Expression of Bcl-XL in Delayed Apoptosis of Canine Neutrophils Induced by Lipopolysaccharide. Res Vet Sci. 2005; 78:183–187. [PubMed: 15563927]
16. Weinmann P, Gaehtgens P, Walzog B. Bcl-Xl- and Bax-Alpha-Mediated Regulation of Apoptosis of Human Neutrophils Via Caspase-3. Blood. 1999; 93:3106–3115. [PubMed: 10216108]
17. Hamasaki A, Sendo F, Nakayama K, Ishida N, Negishi I, Nakayama K, Hatakeyama S. Accelerated Neutrophil Apoptosis in Mice Lacking A1-a, a Subtype of the Bcl-2-Related A1 Gene. J Exp Med. 1998; 188:1985–1992. [PubMed: 9841913]
18. Dzhagalov I, St John A, He YW. The Antiapoptotic Protein Mcl-1 Is Essential for the Survival of Neutrophils but Not Macrophages. Blood. 2007; 109:1620–1626. [PubMed: 17062731]
19. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, Strasser A. Proapoptotic Bcl-2 Relative Bim Required for Certain Apoptotic Responses, Leukocyte Homeostasis, and to Preclude Autoimmunity. Science. 1999; 286:1735–1738. [PubMed: 10576740]
20. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The Proapoptotic Activity of the Bcl-2 Family Member Bim Is Regulated by Interaction With the Dynein Motor Complex. Mol Cell. 1999; 3:287–296. [PubMed: 10198631]

21. Biswas SC, Shi Y, Sproul A, Greene LA. Pro-Apoptotic Bim Induction in Response to Nerve Growth Factor Deprivation Requires Simultaneous Activation of Three Different Death Signaling Pathways. J Biol Chem. 2007; 282:29368–29374. [PubMed: 17702754]
22. Ewings KE, Hadfield-Moorhouse K, Wiggins CM, Wickenden JA, Balmanno K, Gilley R, Degenhardt K, White E, Cook SJ. ERK1/2-Dependent Phosphorylation of BimEL Promotes Its Rapid Dissociation From Mcl-1 and Bcl-XL. EMBO J. 2007; 26:2856–2867. [PubMed: 17525735]
23. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 Signaling Pathway Promotes Phosphorylation and Proteasome-Dependent Degradation of the BH3-Only Protein, Bim. J Biol Chem. 2003; 278:18811–18816. [PubMed: 12646560]
24. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A. BH3-Only Bcl-2 Family Member Bim Is Required for Apoptosis of Autoreactive Thymocytes. Nature. 2002; 415:922–926. [PubMed: 11859372]
25. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB, Henson PM. Modulation of Multiple Neutrophil Functions by Preparative Methods or Trace Concentrations of Bacterial Lipopolysaccharide. Am J Pathol. 1985; 119:101–110. [PubMed: 2984939]
26. Edwards SW, Derouet M, Howse M, Moots RJ. Regulation of Neutrophil Apoptosis by Mcl-1. Biochem Soc Trans. 2004; 32:489–492. [PubMed: 15157168]
27. Chou JJ, Li H, Salvesen GS, Yuan J, Wagner G. Solution Structure of BID, an Intracellular Amplifier of Apoptotic Signaling. Cell. 1999; 96:615–624. [PubMed: 10089877]
28. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. Caspase Cleaved BID Targets Mitochondria and Is Required for Cytochrome c Release, While BCL-XL Prevents This Release but Not Tumor Necrosis Factor-R1/Fas Death. J Biol Chem. 1999; 274:1156–1163. [PubMed: 9873064]
29. O'Neill AJ, Doyle BT, Molloy E, Watson C, Phelan D, Greenan MC, Fitzpatrick JM, Watson RW. Gene Expression Profile of Inflammatory Neutrophils: Alterations in the Inhibitors of Apoptosis Proteins During Spontaneous and Delayed Apoptosis. Shock. 2004; 21:512–518. [PubMed: 15167679]
30. Chen C, Edelstein LC, Gelinas C. The Rel/NF-KappaB Family Directly Activates Expression of the Apoptosis Inhibitor Bcl-x(L). Mol Cell Biol. 2000; 20:2687–2695. [PubMed: 10733571]
31. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ. Expression of the Pro-Apoptotic Bcl-2 Family Member Bim Is Regulated by the Forkhead Transcription Factor FKHR-L1. Curr Biol. 2000; 10:1201–1204. [PubMed: 11050388]
32. Cowburn AS, White JF, Deighton J, Walmsley SR, Chilvers ER. Z-VAD-Fmk Augmentation of TNF Alpha-Stimulated Neutrophil Apoptosis Is Compound Specific and Does Not Involve the Generation of Reactive Oxygen Species. Blood. 2005; 105:2970–2972. [PubMed: 15572588]
33. Andina N, Conus S, Schneider EM, Fey MF, Simon HU. Induction of Bim Limits Cytokine-Mediated Prolonged Survival of Neutrophils. Cell Death Differ. 2009; 16:1248–1255. [PubMed: 19407828]
34. Yan W, Suominen J, Samson M, Jegou B, Toppari J. Involvement of Bcl-2 Family Proteins in Germ Cell Apoptosis During Testicular Development in the Rat and Pro-Survival Effect of Stem Cell Factor on Germ Cells in Vitro. Mol Cell Endocrinol. 2000; 165:115–129. [PubMed: 10940490]
35. Moulding DA, Quayle JA, Hart CA, Edwards SW. Mcl-1 Expression in Human Neutrophils: Regulation by Cytokines and Correlation With Cell Survival. Blood. 1998; 92:2495–2502. [PubMed: 9746790]
36. Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH. Suppression of PMN Apoptosis by Hypoxia Is Dependent on Mcl-1 and MAPK Activity. Surgery. 2000; 128:171–177. [PubMed: 10922988]
37. Saffar AS, Dragon S, Ezzati P, Shan L, Gounni AS. Phosphatidylinositol 3-Kinase and P38 Mitogen-Activated Protein Kinase Regulate Induction of Mcl-1 and Survival in Glucocorticoid-Treated Human Neutrophils. J Allergy Clin Immunol. 2008; 121:492–498. [PubMed: 18036649]
38. Cross A, Moots RJ, Edwards SW. The Dual Effects of TNFalpha on Neutrophil Apoptosis Are Mediated Via Differential Effects on Expression of Mcl-1 and Bfl-1. Blood. 2008; 111:878–884. [PubMed: 17942758]

39. Sawatzky DA, Willoughby DA, Colville-Nash PR, Rossi AG. The Involvement of the Apoptosis-Modulating Proteins ERK 1/2, Bcl-XL and Bax in the Resolution of Acute Inflammation in Vivo. Am J Pathol. 2006; 168:33–41. [PubMed: 16400007]
40. Bauer A, Kirschnek S, Hacker G. Inhibition of Apoptosis Can Be Accompanied by Increased Bim Levels in T Lymphocytes and Neutrophil Granulocytes. Cell Death Differ. 2007; 14:1714–1716. [PubMed: 17585338]
41. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the Fas Pathway of Apoptosis. Cell. 1998; 94:491–501. [PubMed: 9727492]
42. Youle RJ, Strasser A. The BCL-2 Protein Family: Opposing Activities That Mediate Cell Death. Nat Rev Mol Cell Biol. 2008; 9:47–59. [PubMed: 18097445]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

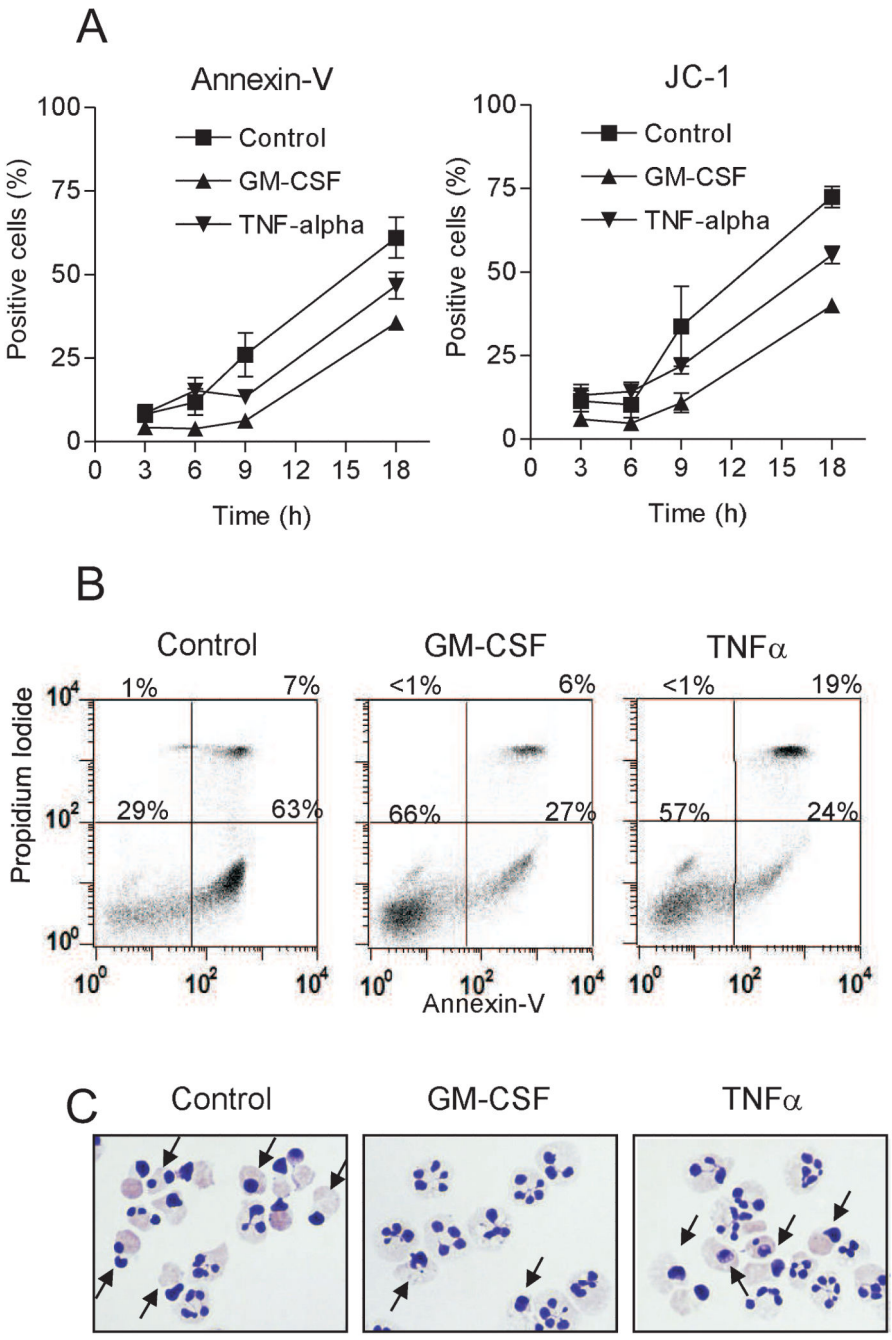

Figure 1. GM-CSF and TNFα inhibit spontaneous neutrophil apoptosis

Human peripheral blood neutrophils were incubated with or without GM-CSF (10 ng/ml) or TNFα (10 ng/ml) for 3-18 hr. (A) Apoptosis was assessed by flow cytometric analysis of either Annexin-V binding or mitochondrial membrane potential (JC-1). GM-CSF and TNFα both promote neutrophil survival and maintain mitochondrial membrane potential compared to time matched controls. Data represent mean ± SEM of data from (n) = 3 independent experiments each conducted in triplicate. (B) representative example of the FACS quandrants analyzing Annexin-v/PI staining of neutrophils incubated with or without GM-

CSF or TNFα. (C) Representative photomicrographs of neutrophils incubated for 18h with or without GM-CSF depicting the morphological features of apoptosis. Arrowheads highlight a higher percentage of neutrophils with condensed nuclei under control conditions compared to GM-CSF and TNFα treated cells.

Europe PMC Funders Author Manuscripts

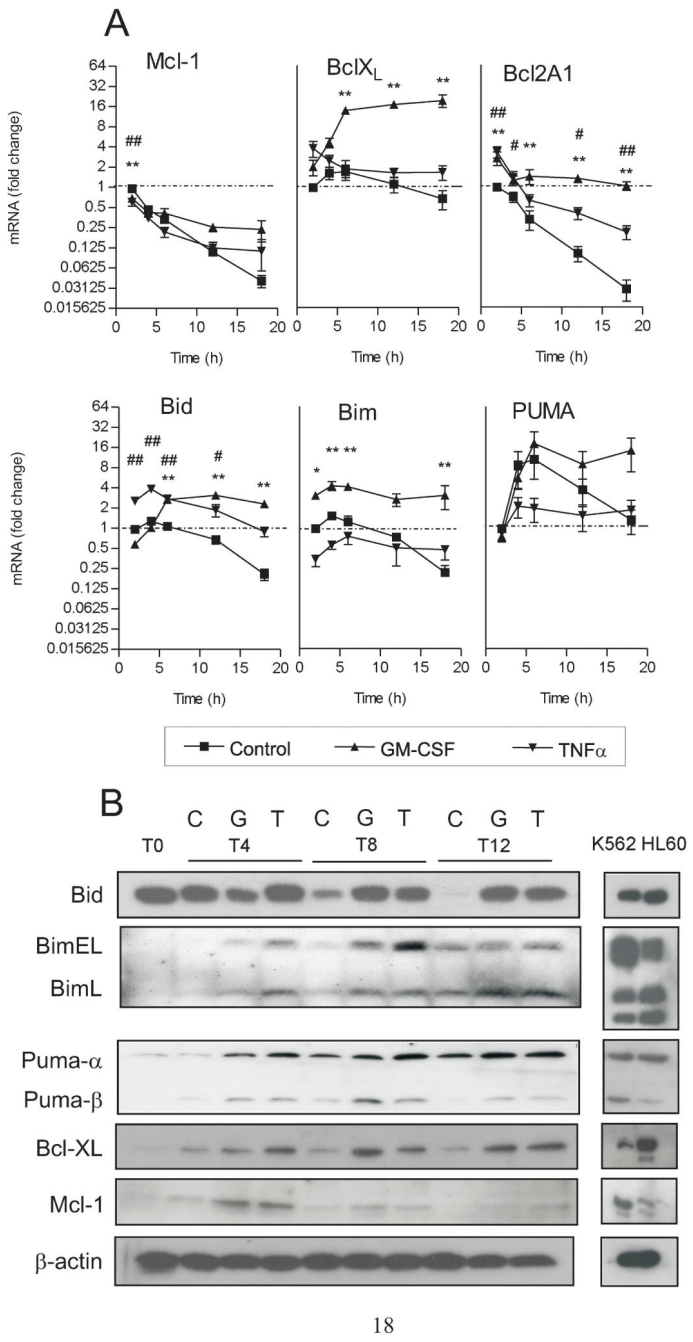

Figure 2. GM-CSF and TNFα regulation of Bcl-2-family members

(A) mRNA profiles for Bcl-2-family members were assessed in isolated peripheral blood neutrophils by qPCR following stimulation with GM-CSF, TNFα or buffer. Gene expression levels at each time point were compared to values obtained in freshly isolated cells and data expressed as relative gene expression (fold change). All gene expression data were normalized to five house-keeping genes to generate ΔΔCT. Data represent mean ± SEM from (n) = 6 separate experiments; statistical analysis by one-way ANOVA, # = TNFα v control, * = GM-CSF v control (*/# $p<0.05$, **/## $p<0.005$). (B) Western blot analysis of

Bcl-2-family members. Neutrophils were cultured for 0, 4, 8 or 12h with and without GM-CSF or TNFα. Western blot analysis confirmed the increases in BimEL expression and Bid stabilization and demonstrate the expression of Bcl-X_L. Control lysates were generated from serum starved K562 and HL-60 cell lines. Data are representative of 3-6 independent experiments.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

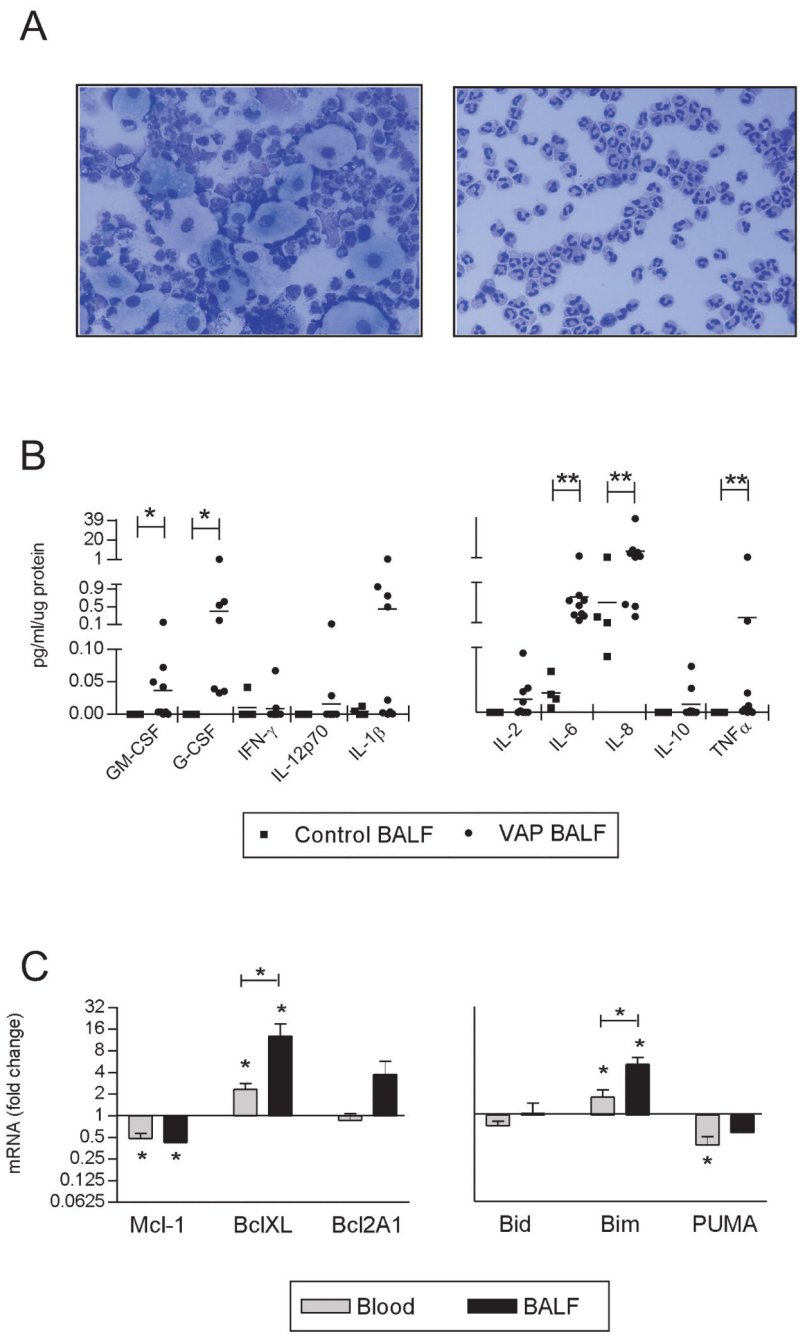

Figure 3. Isolation of BAL and blood neutrophils from patients with VAP and analysis of Bcl-2-family expression

BALF and peripheral blood neutrophils were isolated by RoboSep immuno-magnetic bead separation as detailed. (A) Representative photomicrographs depict local BALF cell population before (Left panel) and after (Right panel) neutrophil purification. (B) ELISA data of inflammatory mediators from the BALF from 8 VAP patients and 4 clinical controls; data are presented as individual values and means. (C) mRNA analysis by qPCR of Bcl-2-family members in neutrophils isolated from BALF and blood from VAP patients referenced

to freshly isolated neutrophils from healthy controls. Data generated from (n) = 8 patients (* $p<0.05$ ** $p<0.005$).

Europe PMC Funders Author Manuscripts

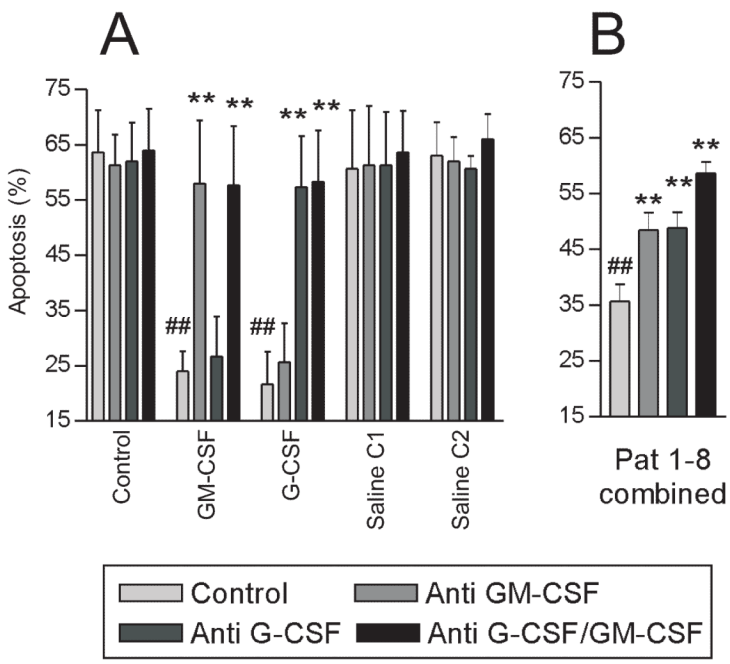

Figure 4. Effect of immuno-depleting GM-CSF and/or G-CSF from BALF derived from VAP patients on neutrophil apoptosis in vitro

Optimisation and specificity of the anti-GM-CSF and anti-G-CSF antibodies was determined using blood neutrophils from healthy subjects incubated over 18h with either GM-CSF or G-CSF. BALF from VAP patients was immuno-depleted of GM-CSF and/or G-CSF using neutralizing polyclonal antibodies, incubated 50:50 (v/v) with purified neutrophils from control subjects for 18 hr and the percentage of apoptosis assessed by FACS analysis of Annexin-V/PI staining. Data shown (means ± SEM) were generated from 8 VAP patients repeated in 3 separate experiments, statistical analysis by one-way ANOVA (* $p<0.05$, ** $p<0.005$ compared to controls; $^{\#\#}$ $p<0.005$ compared to rates of apoptosis observed in the absence of 50% (v/v) BALF.

Europe PMC Funders Author Manuscripts

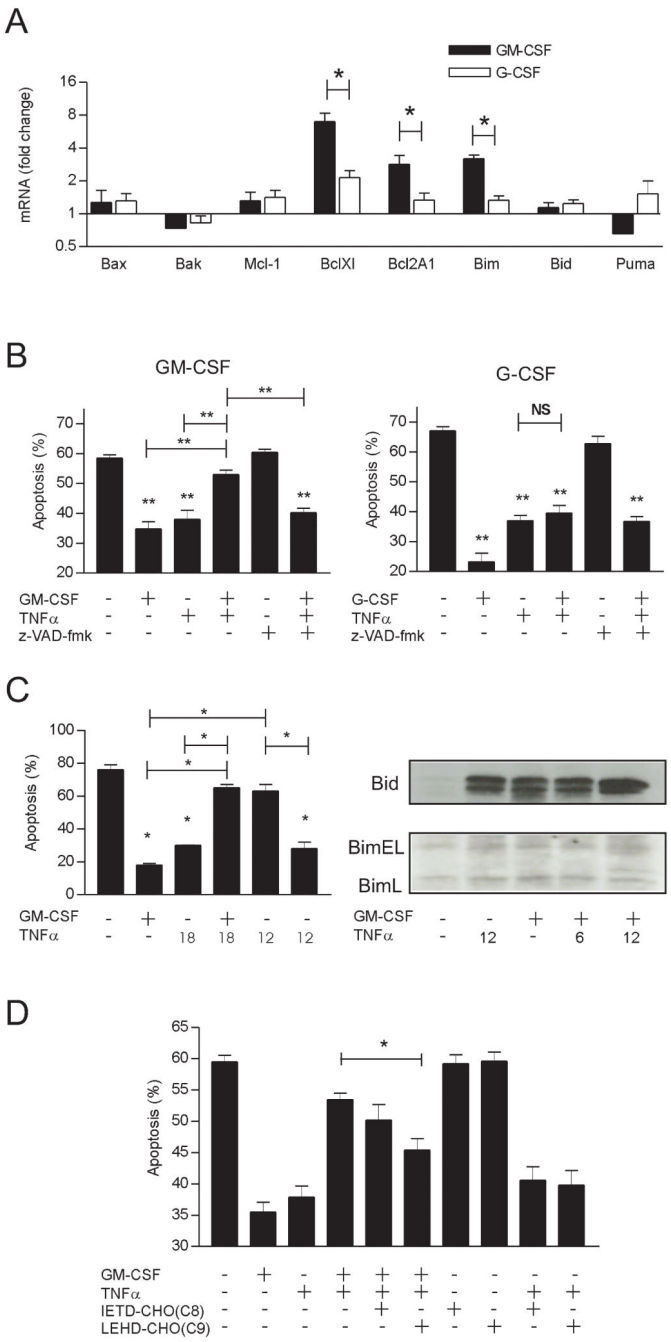

Figure 5. The differential ability of GM-CSF and G-CSF to prime neutrophils for subsequent TNFα-induced apoptosis

(A) Neutrophils incubated with GM-CSF or G-CSF for 6h were analyzed by qPCR to identify differences in the expression of Bcl-2-family members. Data represent mean ± SEM from (n) = 3 independent experiments each performed in triplicate. (B) Neutrophils were incubated with or without GM-CSF or G-CSF for 6h followed by a further 12h with or without TNFα. Apoptosis was assessed by FACS analysis of Annexin-V/PI staining in 5 independent experiments. In those experiments analyzing the role of caspases, z-VAD-fmk was added to the cells 30min prior to TNFα stimulation. (C) Neutrophils were incubated

Europe PMC Funders Author Manuscripts

with buffer or GM-CSF for 6h prior to the addition of TNFα for a further 12h; the selective caspase-8 (IETD-CHO) and caspase-9 (LEHD-CHO) inhibitors were added where indicated 30min prior to the TNFα. Neutrophil apoptosis was assessed by FACS analysis of annexin-v/PI staining in 5 independent experiments, statistical analysis by one-way ANOVA (* $p<0.05$ ** $p<0.005$).

Europe PMC Funders Author Manuscripts

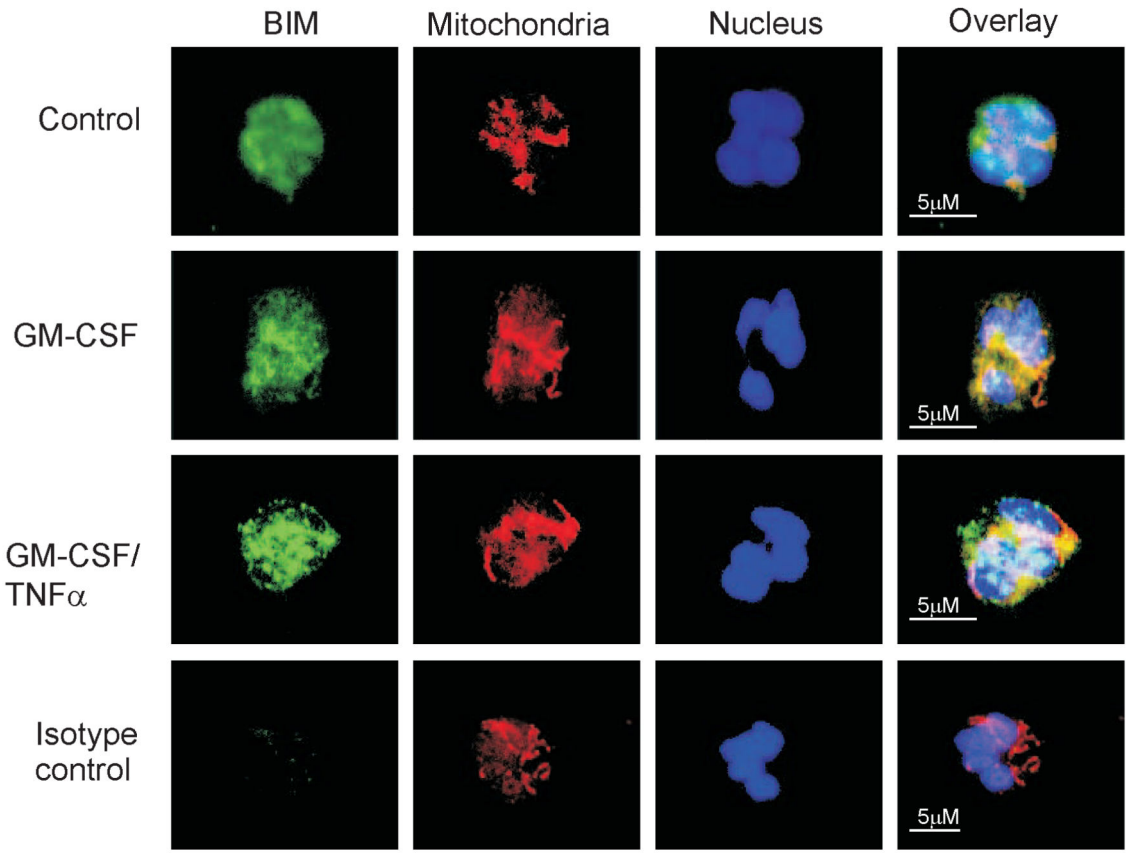

Figure 6. GM-CSF stimulates the re-distribution of Bim towards the mitochondria

Neutrophils were stimulated with or without GM-CSF for 6h followed by 2h incubation with TNFα or buffer; the neutrophils were then incubated with Mitotracker for 30 min prior to fixation with 3.7% paraformaldehyde and staining as detailed. GM-CSF stimulates the formation of intense Bim (green) pyknotic staining that overlays the mitochondrial (red) stain. Additional TNFα stimulation intensifies the Bim (green) pyknotic staining, which continues to cross-over with the mitochondrial (red) staining (see overlay orange/yellow). The photomicrographs are representative of 3 independent experiments.