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IL-6-independent expression of Mcl-1 in human multiple myeloma

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Mcl-1 is a critical antiapoptotic survival factor for human multiple myeloma (MM). We examined the importance of IL-6 for Mcl-1 expression in five MM cell lines and in primary MM cells from 14 patients. While culture of MM.1S cells in IL-6 did induce Mcl-1 expression, four other MM cell lines exhibited high levels of Mcl-1 expression that were unaffected by IL-6. Similarly, Mcl-1 expression in 10 of 14 primary MM isolates was found to be IL-6-independent. An analysis of the mechanisms responsible for IL-6-independent Mcl-1 expression was undertaken. ERK1/2 activity did not influence Mcl-1 expression, distinct from Mcl-1 regulation that occurs during myeloid differentiation from hematopoietic progenitor cells. Inhibition of the PI3K pathway led to growth inhibition of 8226 and ANBL-6 cells without reduction of Mcl-1 levels, and high level Mcl-1 expression was maintained in the absence of activated STAT3. Analysis of the transcriptional activity of 5′-regulatory sequences from the human Mcl-1 gene in MM cells demonstrated high levels of IL-6-independent indicator gene activation as predicted. These data demonstrate that the mechanisms regulating Mcl-1 levels in MM cells are heterogeneous, and are often independent from IL-6 signaling pathways.
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Introduction

Homeostasis within the hematopoietic system is maintained by a carefully orchestrated balance of cell production and the death of mature cells through the process of programmed cell death (Metcalf, 1998). Hematopoietic cell survival is often maintained by the production of survival factors (growth factors, cytokines) that bind to specific receptors and activate signal transduction pathways that prevent activation of the latent core apoptosis apparatus present in all cells (Raff, 1992). Inappropriate expansion of cell numbers is prevented by the removal of environmental trophic signals with subsequent activation of apoptosis. Hematopoietic malignancies are caused by genetic lesions (often translocations and chromosomal instability leading to aneuploidy) that result in a lineage-specific differentiation arrest characterized by the accumulation of cells at a particular stage of development (Alcalay *et al*., 2001). A critical element in the evolution of malignant cells is the capacity to resist apoptotic signals such as deprivation of growth factors, deregulated passage through the G1 restriction point, and DNA damage (Schimmer *et al*., 2001). This acquired resistance to apoptosis also characterizes the difficulty in eradicating tumor cells by conventional therapeutic interventions (Reed, 1995; Johnstone *et al*., 2002).

An important molecular mechanism that promotes the survival of leukemic clones is the increased expression of antiapoptotic members of the Bcl-2 family (Gross *et al*., 1999; Cory and Adams, 2002). Bcl-2, Bcl-XL, and Mcl-1 reside primarily in the mitochondrial outer membrane, where they prevent the release of cytochrome *c* by inhibiting the functions of the apoptosis-inducing Bcl-2 family members including Bax, Bak, and BH3-only proteins (Reed, 1998; Huang and Strasser, 2000). Since the discovery of aberrant regulation of Bcl-2 in follicular lymphomas bearing the t(14;18), overexpression of pro-survival Bcl-2 family members has been shown to play a critical role in the development of many hematopoietic malignancies and their response to therapy (Rowley, 1988; Schimmer *et al*., 2001). In CLL, most clinical isolates express Bcl-2 at high levels, and this alone, or when associated with an increased ratio compared to levels of Bax expression, was predictive of poor outcome (Hanada *et al*., 1993; McConkey *et al*., 1996; Pepper *et al*., 1996; Thomas *et al*., 1996). The mechanisms responsible for high-level Bcl-2 expression in CLL are unknown but rarely involve translocations; in some cases hypomethylation of the promoter region was noted (Raghoebier *et al*., 1991; Hanada *et al*., 1993). A recent study noted that high-level expression of Mcl-1 correlated with failure to attain CR in response to single-agent treatment with alkylating agents or purine nucleosides (Kitada *et al*., 1998). Furthermore, rapid inhibition of Mcl-1 expression by the CDK inhibitor flavopiridol correlated with *in vitro* induction of apoptosis of primary CLL samples (Kitada *et al*., 2000). The potential role of Bcl-2 and Mcl-1 as oncogenes in lymphoid malignancies has been confirmed in transgenic mouse models in which deregulated expression of these transgenes resulted in the expansion of hematopoietic populations with subsequent development of lymphoid tumors (McDonnell *et al*., 1989; Zhou *et al*., 2001).

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Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation in the bone marrow of plasma cells with a low proliferation index and an extended lifespan (Hallek *et al*., 1998; Rajkumar and Greipp, 1999). MM is associated with a variety of chromosomal abnormalities, including translocations to the immunoglobulin heavy chain switch region that occur during antigen-driven B-cell expansion and affinity maturation in the lymph node germinal center (Bergsagel *et al*., 1996). However, although multiple translocation partners have been identified, these do not include Bcl-2 family members (Bergsagel and Kuehl, 2001). Nevertheless, Bcl-2 is highly expressed in malignant plasma cells (Ong *et al*., 1995; Miguel-Garcia *et al*., 1998; Puthier *et al*., 1999c), and Bcl-XL expression was detected more often at the time of patient relapse and correlated with resistance to chemotherapy (Gauthier *et al*., 1996; Tu *et al*., 1998). Furthermore, STAT3 was recently shown to be constitutively activated in primary MM cells, and was shown to induce the upregulation of Bcl-XL in the U266 cell line (Catlett-Falcone *et al*., 1999). Mcl-1 expression has been described in MM cell lines and primary patient isolates, and its expression has been reported to be upregulated by IL-6, a critical MM growth and survival cytokine that is produced by BM stromal cells (Pages *et al*., 1993; Hallek *et al*., 1998; Puthier *et al*., 1999a,b). Recently, we demonstrated that Mcl-1 is required for MM cell survival, and suggested that sustained expression of Mcl-1 by malignant plasma cells at the time of exit from the germinal center could play an important role in the aberrant survival of the malignant clone (Zhang *et al*., 2002). These results were subsequently confirmed by others (Derenne *et al*., 2002).

In the current study, we examine the regulation of expression of Mcl-1 in MM cell lines and in primary $CD138^+$ MM cells, and demonstrate that the response to IL-6 is heterogeneous. We detected significant levels of Mcl-1 expression in all cell lines and patient isolates, and in most cases Mcl-1 levels were not regulated by IL-6. In accord with these data, expression from the human Mcl-1 promoter in MM cells resulted in high levels of expression of the indicator gene in an IL-6-independent manner. Our data indicate that Mcl-1 is constitutively expressed in many MM cells, and that this may be an important factor in the pathogenesis of this disease that, like CLL, depends primarily on failed programmed cell death as opposed to increased proliferation.

Results

Mcl-1 expression in response to IL-6 is heterogeneous in MM cell lines

To examine the role of IL-6 in the regulation of Mcl-1 expression, MM cells were cultured for 24 h in the absence of IL-6, followed by addition of IL-6 for variable time periods. As demonstrated in Figure 1, IL-6 induces the rapid phosphorylation of ERK1/2 (labeled p44/42 in the figures) and tyrosine phosphorylation of STAT3 in all five cell lines. Of note is that for MM1.S, ANBL-6, and OPM-2, the levels of total STAT3 appear to be rapidly induced by IL-6 (in some cases occurring within 5 min of IL-6 addition); the mechanism for this is under investigation. Changes in the level of Mcl-1 expression were then examined. MM.1S cells (panel a) responded to IL-6 in a manner similar to other MM cell lines described in the literature (Puthier *et al*., 1999a,b; Jourdan *et al*., 2000); Mcl-1 was upregulated at 4, 8, and 20 h after addition of IL-6. However, the other four MM cell lines failed to upregulate Mcl-1 in response to IL-6. Mcl-1 was expressed at high levels after the 24 h wash-out period (time 0) even though STAT3 phosphorylation was nearly undetectable and ERK1/2 phosphorylation was either absent or quite low (with the exception of high basal phospho-ERK1/2 in OPM-2 which reflects a mutated FGFR3 in this cell line). After addition of IL-6, there was no change in the level of Mcl-1 expression in any of these four cell lines, either after short (5–30 min) or long (4–20 h) activation periods. These data indicate that IL-6 signaling is active in all five cell lines, but Mcl-1 expression is induced in only one. This IL-6-independent expression of Mcl-1, not previously described in the literature, was therefore studied in more detail.

To further examine the role of serum and IL-6 on Mcl-1 expression, 8226 cells were cultured in decreasing amounts of serum with or without the addition of IL-6. As shown in Figure 2, Mcl-1 expression levels remained high after 24 or 48 h of culture in medium containing 10–0.5% serum, with or without the addition of IL-6. Given the short half of Mcl-1 (30 min to 1 h), culture in 0.5% serum without IL-6 for 48 h would have been expected to substantially lower protein levels if the Mcl-1 gene were dependent on signals emanating from these growth and survival factors. Note that levels of Bcl-XL were also unchanged by these culture conditions.

Changes in the level of Mcl-1 mRNA were examined in MM cells that had been cultured without IL-6 for 24 h followed by its addition for an additional 24 h. As shown in Figure 3, for the 8226, ARP-1, and ANBL-6 cells lines there was no significant change in the level of Mcl-1 expression after the addition of IL-6 (1 or 5 ng/ml). Furthermore, as was seen with protein expression levels, high levels of Mcl-1 mRNA were observed after culture for 24 h in the absence of IL-6 (lanes receiving no IL-6). These data indicate that IL-6 does not regulate the expression of Mcl-1 in these MM cell lines.

The regulation of Mcl-1 gene expression has been investigated in a number of other experimental systems. In cells of different lineages, the Ras/MAPK, Jak2/STAT3, and PI3K pathways have been implicated in the positive regulation of Mcl-1 expression (Craig, 2002). We therefore examined the effects of inhibitors of these pathways on IL-6-independent Mcl-1 expression. As shown in Figure 4a, 8226 cells were cultured in RPMI/10% FCS plus IL-6 followed by the addition of either AG490 (a putative Jak2 inhibitor) or PD98059 (MEK1/2 inhibitor) for the indicated time periods, after which the status of phosphorylation of the target proteins and the expression levels of Mcl-1were determined. In the case of PD98059, ERK1/2 phosphorylation was completely inhibited at the 4, 24, and 48 h time points

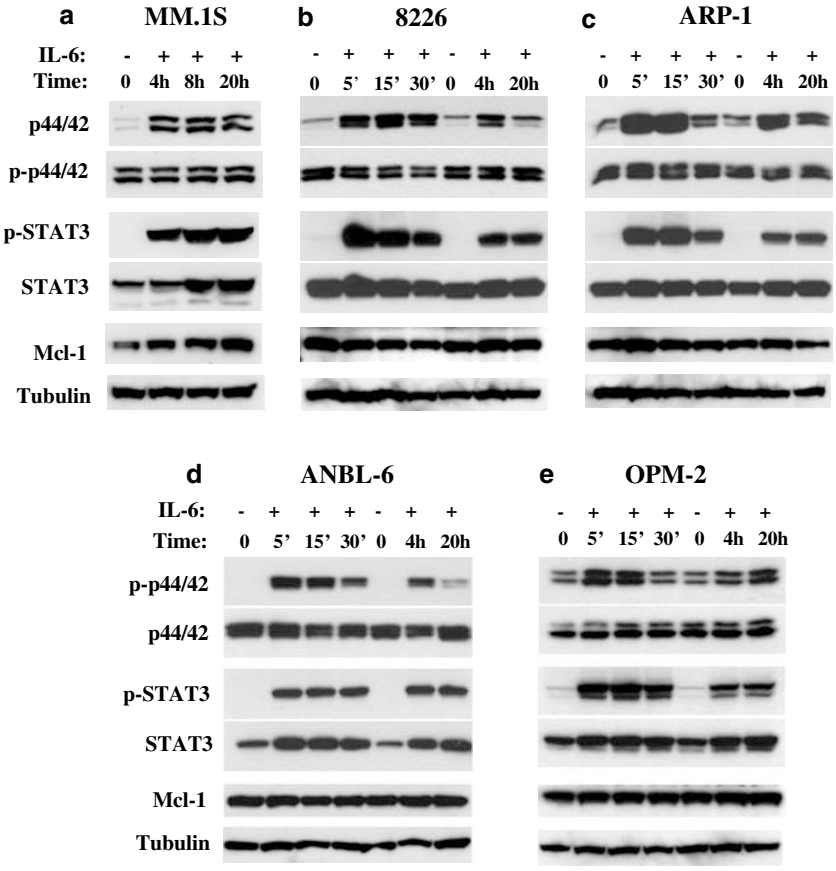

Figure 1 Regulation of Mcl-1 expression by IL-6 is heterogeneous. The indicated MM cell lines were cultured for 24 h in RPMI/10% FCS in the absence of IL-6. IL-6 (5 ng/ml) was then added to the cultures, and protein extracts were prepared at the indicated times. For cells demonstrating no upregulation of Mcl-1 by IL-6 (panels b–e), separate experiments were performed to examine both short-term (0–30 min) or more prolonged signaling effects of IL-6 (0–20 h). Western blot analysis was performed using antibodies directed against phosphorylated forms of ERK1/2 (p-p44/42) or total ERK1/2 (p44/42), tyrosine phosphorylated STAT3 (p-STAT3) or total STAT3, and Mcl-1. Tubulin was used as a control for equal protein loading

without any change in the levels of total ERK1/2 or Mcl-1 expression. Since other investigators have demonstrated that TPA can increase Mcl-1 expression via the ERK1/2 pathway (Townsend *et al*., 1999), we cultured 8226 cells overnight in 0.2% serum in the absence of IL-6, then added TPA for the time periods indicated in Figure 4b. TPA induced ERK1/2 phosphorylation without an increase in the expression of Mcl-1.

Surprisingly, addition of AG490 at high concentration (50 μM) had minimal effect on STAT3 phosphorylation (Figure 4a, lanes 2-4). Other studies from our laboratory indicated that the toxicity of AG490 to MM cell lines was serum dependent; thus AG490 induced growth inhibition but no apoptosis in 10% serum, but apoptosis was observed when cells were cultured in 2% serum. To determine if this was because of down-regulation of Mcl-1 expression, we cultured 8226 and ANBL-6 in medium containing 2% FBS plus IL-6, and tested the effects of AG490 (50 μM) on signaling events, Mcl-1 expression, and cell viability. As shown in Figure 5a, for both 8226 and ANBL-6, AG490 induced a parallel decrease in the levels of phospho-STAT3 and total STAT3 with little or no change in Mcl-1 levels. When cultured in 2% serum, AG490 efficiently induced the apoptosis of 8226 cells (Figure 5b), and it is likely that cell death accounted for the parallel decrease in both total and phosphorylated STAT3 levels (similar results were observed for ANBL-6, not shown). Therefore, we observed no specific inhibition of STAT3 tyrosine phosphorylation by AG490 in our culture system. Consistent with this, AG490-induced death in 8226 cells occurred despite maintenance of Mcl-1 levels (Figure 5a); the mechanism of apoptosis induction under these culture conditions remains to be determined. Further studies have demonstrated that in many of our MM cell lines, IL-6 activates Jak1 and Tyk2, and this may account for the failure of AG490 to block STAT3 phosphorylation (data not shown). The apoptosis induced by AG490 under the low serum conditions (rather that cell cycle arrest noted with 10% serum) is likely because of a more complete inhibition of other tyrosine kinase pathways. However, the effects of AG490 could not be overcome by the addition of either IGF-I or IGF-II (each at 50 ng/ml), which have been demonstrated to be important MM growth factors (data not shown) (Jelinek *et al*., 1997).

To examine the role of the PI3K pathway in regulating Mcl-1 expression, MM cell lines were cultured for

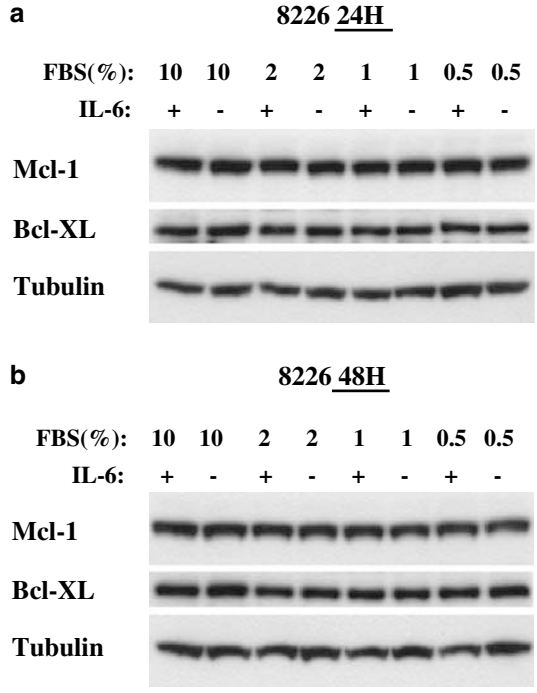

Figure 2 Mcl-1 expression is not regulated by IL-6 or serum in human MM cell line 8226. Cells were cultured in RPMI containing 10, 2, 1 or 0.5% FCS with or without the addition of IL-6 (5 ng/ml) as indicated. After 24 h (panel a) or 48 h (panel b), protein extracts were prepared and subjected to Western blot analysis using antibodies with specificities designated along the left border of each panel

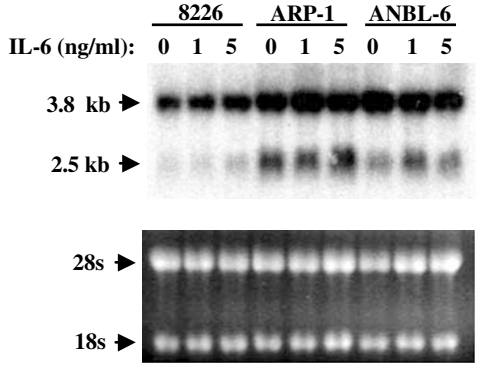

Figure 3 Mcl-1 mRNA levels in MM cell lines are not regulated by IL-6. MM cell lines were washed and then cultured for 24 h in RPMI/10% FCS in the absence of IL-6. Control cells were harvested (0 time point), followed by the addition of IL-6 (1 or 5 ng/ml) as indicated for an additional 24 h. RNA was extracted and Northern blot analysis was performed using an Mcl-1 probe. The ethidium bromide-stained gel demonstrates equal loading of RNA

24 and 48 h in medium containing LY294002 (10 or 20 μM). There was no change in the level of Mcl-1 expression in ANBL-6 or 8226 cells cultured under these conditions (Figure 6a, b). LY294002 was active, as it inhibited the proliferation of both cell lines (Figure 6c). We conclude that Mcl-1 expression in human MM cells does not require PI3K activity, although it is required for cell cycle progression. Surprisingly, culture in

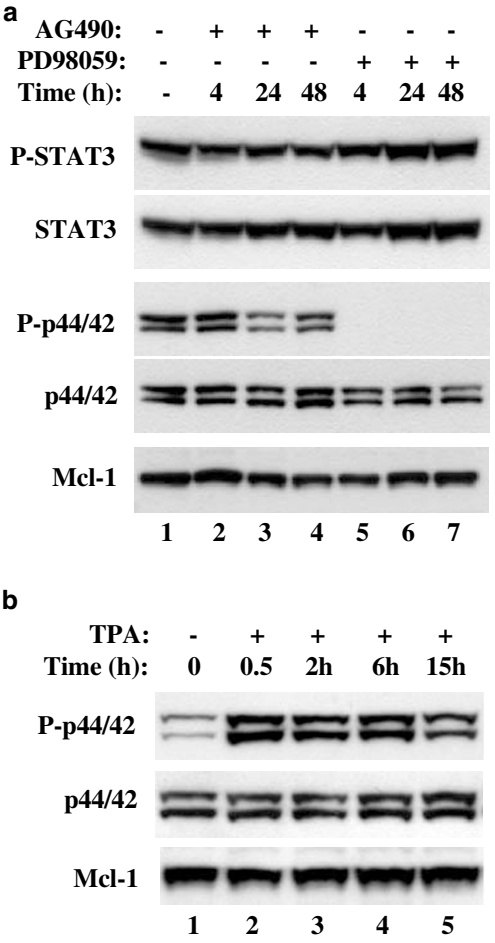

Figure 4 Analysis of regulation of Mcl-1 expression using inhibitors of the Ras/ERK or Jak/STAT pathways. (**a**) 8226 cells were cultured in RPMI/10% FCS plus IL-6 (5 ng/ml) followed by the addition of either PD98059 (50 μM) or AG490 (50 μM). At 4, 24, and 48 h, aliquots of cells were harvested and cell extracts prepared. Western blot analysis was performed using antibodies directed against phosphorylated or total STAT3 and ERK1/2 as described in Figure 1. (**b**) 8226 cells were cultured for 20 h in RPMI containing 0.2% serum without IL-6. TPA (10 ng/ml) was then added, and protein extracts prepared at the indicated times. The activation state of ERK1/2 was determined using a phospho-specific antibody as described in Figure 1. The level of Mcl-1 present in each of the extracts demonstrates no upregulation of Mcl-1 despite activation of the ERK1/2 pathway

LY294002 appeared to increase levels of Bcl-2. This is unusual, as Bcl-2 levels in MM cells are unaffected by most signal transduction inhibitors, and as shown by others, Bcl-2 is not upregulated by IL-6 (Puthier *et al*., 1999a,b; Jourdan *et al*., 2000).

Regulation of Mcl-1 expression in primary MM cells

Previous studies of a single primary MM isolate (a plasma cell leukemia) showed that IL-6 induced a dramatic increase in Mcl-1 protein levels (Puthier *et al*., 1999b). To examine this important question using a larger number of patient samples, we purified mononuclear cells from the BM of MM patients and cultured the cells for 18 h in the absence of IL-6. Cells were then

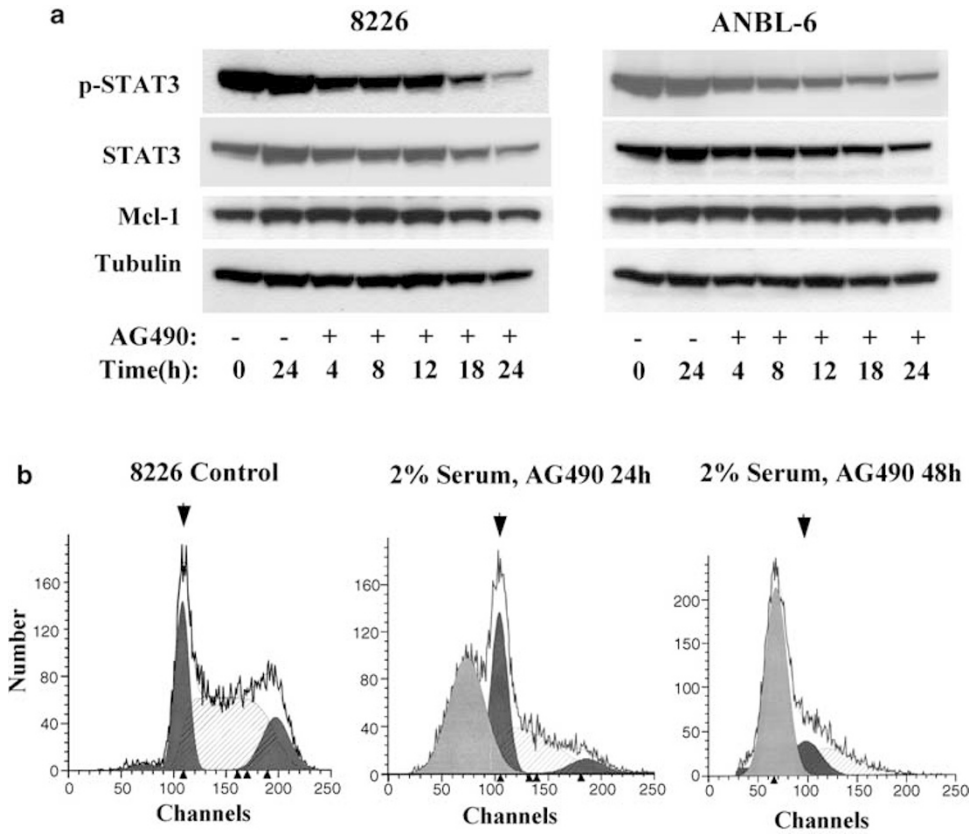

Figure 5 Induction of apoptosis of MM cell lines by AG490 is serum dependent. (**a**) MM cell lines 8226 and ANBL-6 were cultured in RPMI/2% FCS plus IL-6 for 0–24 h with or without the addition of AG490 (50 μM) as indicated. At the times shown, cell extracts were prepared and Western blot analysis performed to determine the activation state of STAT3, total STAT3 levels, and changes in the expression of Mcl-1. (**b**) Cell cycle analysis was performed on 8226 cells cultured for 24 h in RPMI/2% FCS plus IL-6 (control) or in this same medium plus AG490 (50 μM) for 24 or 48 h. Cells were harvested, fixed, and stained with propidium iodide. The arrowhead indicates the position of the G1 peak (2N DNA content)

separated into $CD138^+$ and $CD138^-$ populations and cultured with or without IL-6. Protein lysates were prepared at the indicated times, and the levels of Mcl-1 and other Bcl-2 family members were determined. In some cases sufficient protein was available from the $CD138^+$ fraction to perform a more detailed analysis of IL-6-induced signaling pathways, and when possible both $CD138^+$ and $CD138^-$ populations were studied in parallel. Three cases of the more detailed signal transduction analyses are shown in Figure 7. Cells from patient #1 demonstrated activation of the ERK1/2 and STAT3 pathways after the addition of IL-6, although the basal level of phosphorylation of these proteins (time 0) was high compared to other patients. In addition, in extracts from the $CD138^+$ population, residue Ser727 of the STAT3 activation domain appeared to be constitutively phosphorylated. These data suggest that there is an activated MAPK pathway in these cells, perhaps because of mutation of Ras or FGFR3; this is currently under study. Of importance was the observation that expression of Mcl-1 was quite high in the $CD138^+$ population even after 18 h of culture without IL-6, and that this level did not increase during a subsequent 24 h culture in the presence of IL-6. Levels of Bcl-2 and the low-level expression of Bcl-XL were also unchanged.

Patients 2 and 3 exhibited similar patterns of ERK1/2 and STAT3 activation in response to IL-6. For unclear reasons, very low levels of total ERK1/2 were noted in the $CD138^+$ population from patient 2 (observed in two independent experiments). Mcl-1 levels in $CD138^+$ cells from patient 2 showed no increase in response to IL-6. In contrast, Mcl-1 levels in patient 3 were elevated two-fold after 8 and 24 h of culture in the presence of IL-6. Thus for all three patients, culture of purified primary MM cells in IL-6 lead to activation of the appropriate signaling pathways, yet in two of three samples we did not observe any increase in the expression level of Mcl-1. In each case, the basal level of Mcl-1 expression was quite high even after culture for 18 h in the absence of IL-6.

Smaller numbers of $CD138^+$ cells were available from 11 other patients, and the analysis of the effects of IL-6 on protein expression was therefore limited to Mcl-1 and other antiapoptotic Bcl-2 family proteins (Figure 8). $CD138^+$ (and in some cases equal numbers of $CD138^-$ cells) were cultured for 18 h in the absence of IL-6, followed by the addition of IL-6 to half the cells and

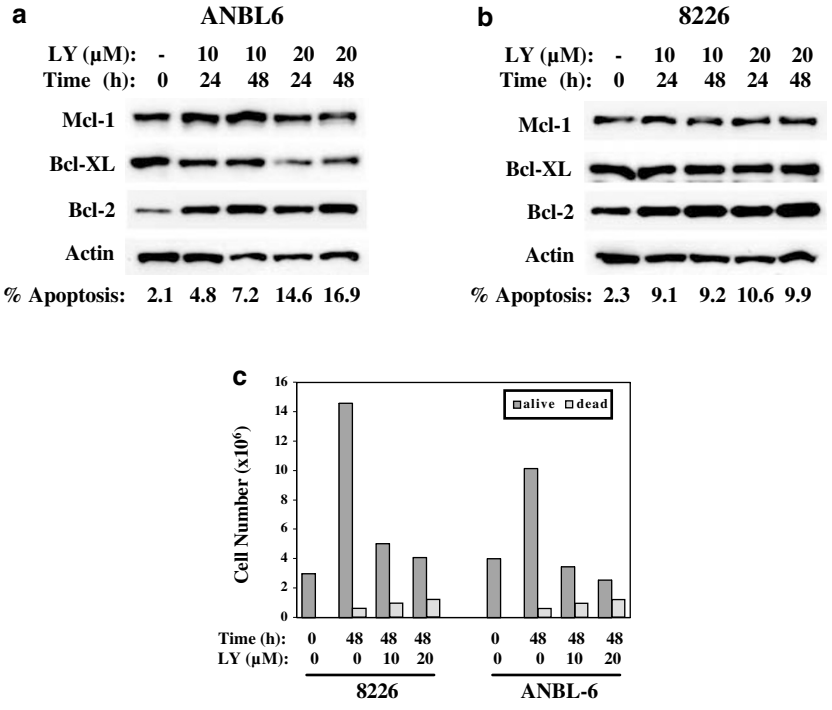

Figure 6 The PI3K inhibitor LY294002 inhibits MM cell growth, but not expression of Mcl-1. Panels a and b: ANBL-6 cells were cultured in RPMI/10% FCS with IL-6 (1 ng/ml), whereas 8226 cultures did not receive IL-6. Cells were then cultured with 10 or 20 μg/ml of LY294002 for 24 or 48 h, and cells were harvested for preparation of protein extracts and determination of per cent apoptosis (Hoechst 33342 staining of cytospins; at least 200 cells were evaluated for each time point). Western blot analysis was performed using the antibodies indicated along the left margin. Panel c: cells were cultured in the medium described above with or without the addition of 10 or 20 μM LY294002 for 48 h. Cells were counted and viability was determined by trypan blue exclusion

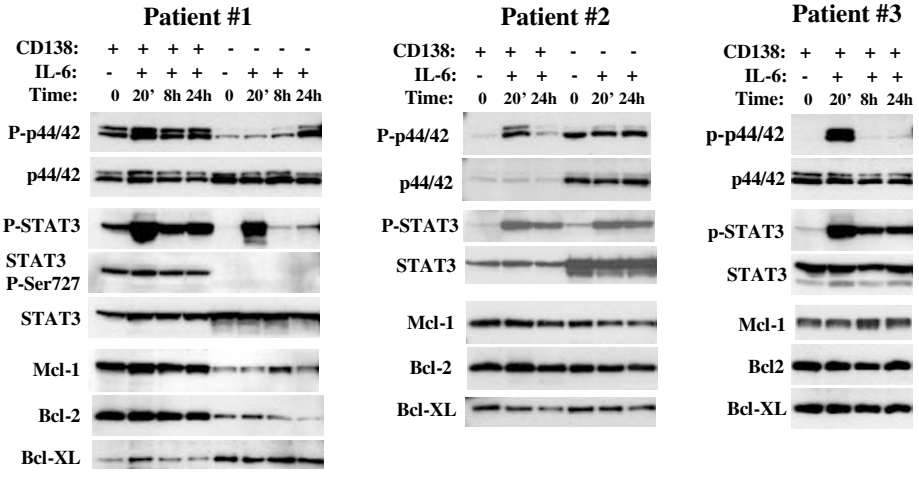

Figure 7 IL-6-dependent signal transduction profiles and expression levels of Bcl-2-family proteins in purified CD138$^+$ cells from bone marrow of MM patients. Mononuclear cells from BM aspirates were cultured for 18 h in RPMI/10% FCS and then separated into CD138$^+$ and CD138$^-$ fractions. IL-6 was added to the cultures (controls received no IL-6), and protein extracts were prepared at the times indicated. Western blot analysis was performed using a panel of antibodies with specificities designated along the left margin. For patient 1, the level of STAT3 phosphorylation at Ser727 of the activation domain was determined. For patient 3, analysis was performed on the CD138$^+$ population only

culture for an additional 24 h. Note that in these samples, as well as those in Figure 7, the level of Bcl-2 within each cell population did not vary (i.e. was independent of IL-6), and was therefore used as an indicator of equal protein loading. This is in agreement with previously published reports demonstrating that Bcl-2 levels are not regulated by IL-6 (Puthier *et al*., 1999a,b; Jourdan *et al.,* 2000). Analysis of patient 7 demonstrated a two-fold upregulation of Mcl-1 protein levels after culture in IL-6. CD138$^+$ cells from patients 6

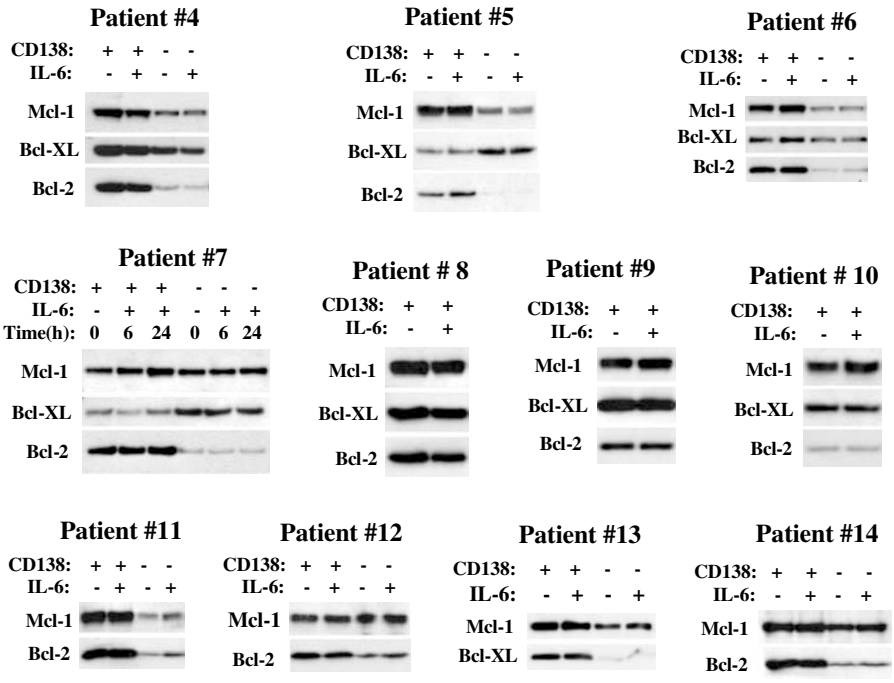

Figure 8 Regulation of Mcl-1 expression by IL-6 in a panel of 11 freshly isolated MM specimens. Mononuclear cells isolated from BM aspirates of MM patients were cultured in RPMI/10% FCS for 18 h and then fractionated into $CD138^+$ and $CD138^-$ populations; in some cases only the $CD138^+$ cells were analysed. Cells were cultured for an additional 24 h with or without IL-6 (5 ng/ml), and protein extracts were prepared and subjected to Western blot analysis using antibodies directed against antiapoptotic Bcl-2 family members

and 10 also exhibited a small increase in Mcl-1 levels. The remaining eight patient samples demonstrated high levels of *de novo* Mcl-1 expression that did not increase after culture in IL-6. These data show that Mcl-1 was expressed in all 14 primary MM isolates tested, and in 10 of these (71%) its expression was not increased by IL-6. However, in four of 14 cases there was an approximate 1.5–2-fold induction that could have significant protective effects in the face of apoptotic stimuli.

This patient survey is too small to allow meaningful correlations to be made between clinical parameters and response of Mcl-1 expression to IL-6. However, we noted that all four patients demonstrating upregulation of Mcl-1 (patients 3, 6, 7, and 10) had not been treated with high-dose chemotherapy and autologous stem cell transplantation (ASCT). Of the remaining 10 patients whose Mcl-1 levels were not upregulated by IL-6, five had relapsed after ASCT and five had not yet been transplanted and were minimally treated (two or fewer regimens). It remains to be determined if expression of Mcl-1 becomes IL-6-independent after high-dose therapy; analysis of patients pre- and post-transplant will be required to answer this question.

Mcl-1 regulation in MM cells grown in serum-free conditions

It has been reported that IL-6-mediated upregulation of Mcl-1 is an important survival pathway when MM cell lines are cultured in serum-free medium to remove the confounding effects of other survival factors such as IGF-1 (Jourdan *et al*., 2000). In those studies, culture of the XG-series of IL-6-dependent cell lines in 1% bovine serum albumin (BSA)/RPMI resulted in the rapid induction of apoptosis (i.e. 60% annexin V positivity at 2–6 h), while addition of IL-6 conferred some protection. This correlated with an upregulation of Mcl-1 by IL-6 (Jourdan *et al*., 2000). We performed similar experiments using our panel of MM cell lines. ARP1, ANBL-6, and 8226 cells were cultured in RPMI containing 1% BSA with or without IL-6 and the per cent apoptosis was determined. As shown in Table 1, we observed low levels of apoptosis after 24 h and this was not influenced by IL-6. Cell cycle analysis of cells cultured under these conditions demonstrated cell cycle

Table 1 Survival of MM cells cultured in 1% BSA ± IL-6

Cell line	*Culture conditions*			
	FBS (−) IL-6	*FBS (+) IL-6*	*BSA (−) IL-6*	*BSA (+) IL-6*
ANBL-6	7.3 ± 1.4	7.7 ± 1.5	9.9 ± 1.9	11.5 ± 1.7
8226	1.2 ± 0.6	2.9 ± 1.2	7.6 ± 1.5	5.0 ± 1.4
ARP-1	3.9 ± 1.3	6.4 ± 1.5	6.6 ± 1.7	6.6 ± 1.7

MM cell lines were cultured in RPMI medium containing 10% FBS with or without IL-6 (1 ng/ml), or in RPMI containing 1% BSA with or without IL-6, as indicated. After 24 h, cytospin preparations were subjected to TUNEL assay with Hoechst 33342 counterstain. At least 200 cells were counted at each data point, and the per cent of apoptotic cells was determined

arrest with <10% apoptosis at 24 h, and Western blots showed little change in the level of Mcl-1 protein (data not shown). To further assess the survival role of IL-6 under serum-free conditions, primary CD138$^+$ cells were subjected to a similar analysis. Cells were cultured in 10% FBS or 1% BSA with or without IL-6 as indicated in Figure 9. Protein expression levels and the per cent apoptosis were determined after 24 h. Culture in 1%BSA led to a 50% decrease in Mcl-1 and Bcl-XL levels compared with culture in 10% FBS, with a small but significant increase in the frequency of apoptosis. This was not surprising given the role of serum survival factors (e.g. IGF-1 and others) in maintaining cell viability. Importantly, the addition of IL-6 did not effect Mcl-1 expression levels during culture in FBS or BSA, and did not rescue cells from apoptosis induced by culture in 1% BSA. We were therefore unable to confirm previous observations of the rapid induction of apoptosis of MM cells cultured in serum-free medium (Jourdan *et al*., 2000), and we did not detect a role for IL-6 in regulating Mcl-1 levels in cell lines or primary cells cultured under these conditions.

Analysis of the human Mcl-1 promoter in MM cell lines

The data thus far presented support the hypothesis that in many MM cell lines and primary MM cell isolates, Mcl-1 levels are not regulated by IL-6. Since Mcl-1 is regulated at the transcriptional level in many systems (Craig, 2002), our data led to the prediction that ectopic expression of the human Mcl-1 promoter in MM cell lines should reproduce the high levels of constitutive, IL-6-independent expression. To test this hypothesis, ARP1 cells were transfected with plasmid constructs containing 5′-regulatory sequences from the human Mcl-1 promoter cloned upstream of the luciferase indicator gene (Akgul *et al*., 2000b); the pGL3 basic vector and SV40-luciferase constructs served as negative and positive controls, respectively. As shown in Figure 10a,

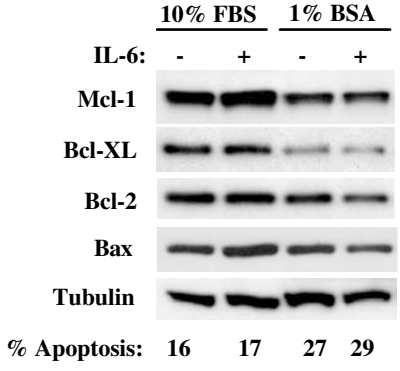

Figure 9 Regulation of Mcl-1 expression and induction of apoptosis under serum-free culture conditions. Primary CD138$^+$ MM cells were isolated from a BM aspirate and cultured for 24 h in RPMI plus 10% FBS or 1% BSA with or without IL-6 (1 ng/ml) as indicated. Cells were then harvested and Western blot analysis was performed. An aliquot of cells from each experimental group was analysed by TUNEL/Hoechst staining and the corresponding per cent apoptosis was determined for each sample

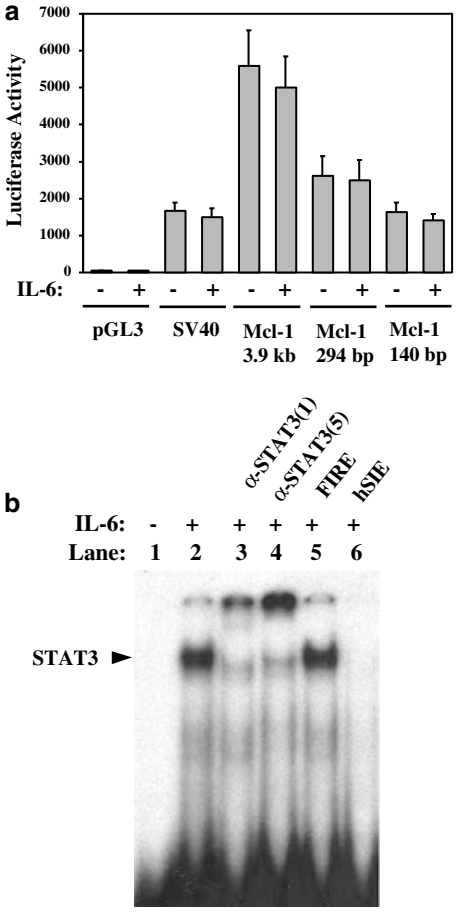

Figure 10 The human Mcl-1 promoter is active in human MM cells, but is not regulated by IL-6. (**a**) ARP1 cells were cultured for 24 h in RPMI/10% FCS in the absence of IL-6. Cells were transfected with luciferase expression constructs with no promoter (pGL3), the SV40 early promoter/enhancer, or differing lengths of the Mcl-1 5′-regulatory region as indicated; each transfection included a small amount of the pRL-TK plasmid to control for transfection efficiency. IL-6 (5 ng/ml) was added to one of two parallel cultures 6 h after transfection, and the cells were cultured for an additional 18 h. Cells were then harvested and dual luciferase/Renilla assays were performed. The data represent the mean of three independent experiments; error bars show the standard deviation. (**b**) Activation of STAT3 by IL-6 in ARP1 cells as demonstrated by EMSA. ARP1 cells were cultured as described above, and nuclear extracts were prepared from cells cultured without (lane 1) or with IL-6 (5 ng/ml for 30 min; lanes 2–6). STAT3 DNA binding was detected using ^{32}P-labeled hSIE oligonucleotide. In lanes 3 and 4, 1 or 5 μl of anti-STAT3 antibody was used to specifically supershift complexes containing STAT3. In lanes 5 and 6, cold-competition assays were performed using a 100-fold molar excess of nonspecific (FIRE) or specific (hSIE) competitor peptide

constructs containing 3.9 kb of Mcl-1 5′-regulatory sequences induced high levels of luciferase expression; in fact the levels are 2–3-fold higher than those observed using the SV40 early promoter/enhancer construct. When the Mcl-1 regulatory sequences were shortened to 294 (*Xho*I/H3) and 140 bp (*Pvu*II/H3), there was a progressive decrease in luciferase expression, although both short constructs retained significant activity. For all three Mcl-1 promoter constructs, addition of IL-6

during the final 24 h of culture did not lead to increased luciferase expression. Electrophoretic mobility gel shift analysis (EMSA) demonstrated that STAT3 activation in ARP1 was IL-6-dependent and quite robust. Specificity was demonstrated by cold competition with specific (hSIE) or irrelevant oligonucleotides (FIRE), and by supershift with an antibody directed against STAT3. These data demonstrate that the human Mcl-1 promoter is very active in MM cells, and that this high level of expression is IL-6-independent and occurs in the absence of detectable STAT3 DNA-binding activity.

Discussion

There is accumulating experimental and clinical data to support the hypothesis that Mcl-1 plays a critical role in the survival of malignant hematopoietic cells. This is most clearly evident for CLL, MM, and AML (Kaufmann *et al*., 1998; Kitada *et al*., 2000; Zhang *et al*., 2002). Prevention of mitochondrial outer membrane permeabilization (MOMP) is a key regulatory feature of antiapoptotic Bcl-2 family members, and the sum of the levels of Bcl-2, Bcl-XL, and Mcl-1 in the outer membrane may be a critical determinant of resistance to apoptosis (Reed, 1998; Gross *et al*., 1999; Goldstein *et al*., 2000; Martinou and Green, 2001; Cory and Adams, 2002). Mcl-1 has unique properties compared to Bcl-2 and Bcl-XL including the very short half-life of its mRNA and protein (Akgul *et al*., 2000a; Chao *et al*., 1998; Kozopas *et al*., 1993). Since changes in the levels of Mcl-1 in response to cytokines, TPA, and other signals are thought to be regulated primarily at the level of transcription (Craig, 2002), environmental signals that alter the rate of Mcl-1 transcription would be expected to rapidly reset the antiapoptotic rheostat within the tumor cell. Interventions that inhibit Mcl-1 synthesis, even for short periods, would be expected to result in a significant reduction in Mcl-1 protein levels and render tumor cells vulnerable to apoptosis (Kitada *et al*., 2000; Gojo *et al*., 2002; Zhang *et al*., 2002). It is therefore important to identify trophic signals or cell autonomous pathways that maintain Mcl-1 levels above the apoptotic threshold. In this paper, we examined the regulation of Mcl-1 expression in MM, a tumor dependent more on resistance to apoptosis than upon aberrant cell proliferation, and in which Mcl-1 has been shown to play a critical role in tumor cell survival (Derenne *et al*., 2002; Zhang *et al*., 2002).

Our data extend the work of other investigators by confirming that Mcl-1 protein is expressed in all MM cells thus far examined, and that Mcl-1 levels are upregulated by IL-6 in some MM cell lines and primary MM cells (Puthier *et al*., 1999a,b; Jourdan *et al*., 2000). However, our data demonstrate that IL-6-independent expression of Mcl-1 is often observed in MM cells. This is not secondary to nonfunctional IL-6 signaling pathways, as activation of ERK1/2 and STAT3 was always observed after the addition of IL-6 to myeloma cultures. Overall four of five cell lines and 10 of 14 primary MM isolates exhibited IL-6-independent Mcl-1 expression. The number of patient samples analysed in this study was too small to draw meaningful correlations between the extent of previous treatment and Mcl-1 responsiveness to IL-6. However, the data suggest that cells from patients who had not received ASCT may be more dependent on IL-6, as they tended to exhibit IL-6 responsiveness for Mcl-1 gene regulation. Culture of MM cell lines in serum-free medium containing 1% BSA failed to replicate the massive, rapid induction of apoptosis or the decline in Mcl-1 levels as described by others (Jourdan *et al*., 2000). Although culture of primary MM cells in 1% BSA did modestly increase the frequency of apoptosis associated with a decline in Mcl-1, neither event was rescued by IL-6. The disparate data may reflect biologic differences in the cell lines used by each laboratory.

The high levels of Mcl-1 mRNA and protein observed in MM cells are regulated primarily at the level of transcription of the Mcl-1 gene. This is indicated by the fact that Mcl-1 mRNA and protein both have a short half-life in MM cells (Gojo *et al*., 2002; Zhang and Fenton, unpublished data), thus requiring efficient gene transcription to maintain steady-state levels. This is supported by our transfection studies in which 3.9 kb of Mcl-1 5′-regulatory sequences induced levels of luciferase expression that were significantly higher than that induced by the SV40 early region promoter/enhancer. Promoter constructs containing 294 or 140 bp were less active, unlike a previous study in U937 cells showing that overall promoter activity as well as GM-CSF and TPA-responsiveness were encoded by the 294 bp construct without contribution from upstream sequences (Akgul *et al*., 2000b). Addition of IL-6 to transfected MM cells did not induce higher levels of indicator gene expression from any of the Mcl-1 promoter constructs. Thus, activity of the Mcl-1 5′-regulatory region mimics the IL-6-independent expression of the endogenous Mcl-1 gene, and will provide a useful model to map important cis-acting regulatory elements.

How do we explain the difference in IL-6-responsiveness of Mcl-1 expression observed in various MM cell lines and primary MM isolates? MM is known to be a heterogeneous disease based on clinical features (Hallek *et al*., 1998; Rajkumar and Greipp, 1999), differences in chromosomal abnormalities (Tricot *et al*., 1997; Fonseca *et al*., 1999), and in the global expression patterns observed in large numbers of MM patients (Zhan *et al*., 2002). Transcriptional activators/repressors, chromatin remodeling activities, and signal transduction proteins were included in the groups of most significantly down- or upregulated genes in MM as compared with normal bone marrow plasma cells; great variation was also observed between subgroups of MM (Zhan *et al*., 2002). Thus, as with leukemias in general, MM is a disease characterized by transcriptional deregulation, with a wide spectrum of gene expression patterns observed. Given this genetic heterogeneity, we believe it would be highly improbable that expression of Mcl-1 in response to IL-6 would be the same in all MM cells.

The data indicate that in cells exhibiting IL-6-independent Mcl-1 expression, Mcl-1 expression is

maintained by mechanisms that do not depend upon the ERK1/2, STAT3, or PI3K pathways normally activated by IL-6. Data from our inhibitor studies support this contention. This is in contrast to studies using a variety of other cell types in which the Ras/ERK, Jak/STAT, and/or PI3K pathways play an important role in regulating Mcl-1 gene expression in response to CSFs and cytokines (Craig, 2002). For instance, in human hematopoietic progenitor and myeloid cell lines, Mcl-1 was regulated by the MAPK and PI3K pathways (Townsend *et al*., 1999; Huang *et al*., 2000; Schubert and Duronio, 2001). In human neutrophils, GM-CSF effects on Mcl-1 were blocked by PI3K inhibitors and Mcl-1 antisense oligonucleotides (Moulding *et al*., 2000; Epling-Burnette *et al*., 2001), while in human and murine macrophages, the PI3K pathway appeared to regulate Mcl-1 levels (Helgason *et al*., 1998; Di Cristofano *et al.,* 1999; Liu *et al*., 2001). In agreement with others, our data strongly mitigate against a role for ERK1/2 in the regulation of Mcl-1 expression in MM cells (Puthier *et al*., 1999a). The role of the PI3K pathway, which can be activated in MM by deletion of PTEN (Ge and Rudikoff, 2000; Hyun *et al*., 2000), was also tested in our system. At doses of LY294002 that inhibited cell proliferation, there was no decline in Mcl-1 levels in the ANBL-6 or 8226 cell lines. Previously, we reported that these cells exhibited only low levels of Akt kinase activity in response to IL-6, and suggested that other pathways downstream of PI3K and PDK1 were inhibited by LY294002 and were likely to be important for cell cycle progression in MM cells (Zhang and Fenton, 2002). Experiments performed by others using the MM.1S myeloma cell line did show an increase in Akt Ser473 phosphorylation in response to IL-6, although, as with our data, inhibition with LY294002 led to G1 cell cycle arrest without the induction of apoptosis (Hideshima *et al*., 2001). Mcl-1 levels were not evaluated in that study. We were unable to demonstrate a specific inhibition of the STAT3 pathway using AG490 (Meydan *et al*., 1996; Bright *et al*., 1999; Puthier *et al*., 1999a); however, Mcl-1 expression is maintained when cells are grown in the absence of IL-6 and activated STAT3 is undetectable.

These data underscore the differences in expression of Mcl-1 in human MM compared to other systems, and are consistent with the hypothesis that the mechanism of Mcl-1 gene regulation in MM is distinct. In some experimental systems, Mcl-1 levels increase transiently during periods of cell differentiation, presumably acting to suppress apoptosis during particularly vulnerable transitions for these cells (Kozopas *et al*., 1993; Sordet *et al*., 1999; Moulding *et al*., 2000; Craig, 2002). In MM cell lines and primary cells, Mcl-1 levels are maintained at a high level, and we observe no evidence for a transient activation of Mcl-1. Whether a more regulated expression of Mcl-1 occurs during the differentiation of normal plasma cells in the LN germinal center is unclear, however, it is possible that Mcl-1 plays an important role in determining the lifespan of short- and long-lived plasma cells (Slifka *et al*., 1998). A future goal will be to determine which signal transduction pathways regulate IL-6-dependent and IL-6-independent expression of Mcl-1 in human MM. We have begun this analysis with our studies of cis-acting promoter/enhancer sequences in the human Mcl-1 promoter with the aim of identifying key regulatory elements and the transcription factors that bind them in human MM cells. Candidates include factors normally involved in plasma cell differentiation (e.g. XBP-1, Blimp-1, or IRF-4; Calame, 2001) or factors deregulated by the neoplastic process (Zhan *et al*., 2002). By understanding the molecular mechanisms governing expression of Mcl-1 in different subsets of MM, it may be possible to develop disease-specific therapeutics to target those factors required for the expression of this critical survival factor.

Materials and methods

Cell lines

The MM cell lines 8226, ARP-1, OPM-2, and ANBL-6 were kindly provided by Dr Guido Tricot (Arkansas Cancer Research Center, Little Rock, AR, USA) and Dr Diane Jelinek (Mayo Clinic, Rochester, MN, USA). MM.1S cells were provided by Dr Steven Rosen (Northwestern University, Chicago, IL, USA). Cells were cultured in RPMI-1640 medium supplemented with 100 IU/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% FBS (all from Life Technologies, Inc., Gaithersburg, MD, USA), and 1 ng/ml recombinant human IL-6 (R&D Systems, Minneapolis, MN, USA). 8226 encodes mutated K-Ras (G12A), is resistant to dexamethasone, and proliferates in the absence of IL-6. ANBL-6 encodes wild-type Ras and requires IL-6 for its proliferation and survival. ARP1 proliferates more slowly in the absence of IL-6 (i.e. it is IL-6-responsive but not dependent); like the other lines, ARP1 has no functional p53 pathway. OPM-2 contains a t(4;14), which encodes an activated FGF receptor 3. PD98059 (Calbiochem-Novabiochem Corp., San Diego, CA, USA) was dissolved in DMSO (50 mM stock). At the concentration used in most experiments in this paper (50 μM), PD98059 is a specific inhibitor of MEK1 and MEK2 with no activity against 16 other cellular kinases (Alessi *et al*., 1995). The tyrphostin AG490 was obtained from Alexis Biochemicals Corp., San Diego, CA, USA and stored as a 50 mM stock.

Western blot analysis

Cells were lysed on ice in modified RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) NP-40, 0.25% Na-deoxycholate, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM NaF, and 2 μg/ml leupeptin and aprotinin. Protein concentrations were determined in triplicate using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). Cell lysates that were size fractionated on 10% Tris-glycine gels or NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). For cell lines, 50 μg of protein was loaded, and for patient samples 10–20 μg was loaded per well. Antibodies used in this study were: phospho-p44/42 MAP kinase antibody, p44/42 MAP kinase antibody, phospho-STAT3 antibody, and STAT3 antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-Mcl-1 (rabbit polyclonal Ab S-19; band size 40 kDa) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; anti-Bcl-XL (26 kDa) rabbit polyclonal Ab (Transduction Laboratories,

Lexington , KY, USA); anti-human Bcl-2 (26 kDa) mouse monoclonal Ab clone 124 (Upstate Biotechnology, Lake Placid, NY, USA); anti-tubulin Ab-4 (NeoMarkers, Fremont, CA, USA); anti-actin (I-19, Santa Cruz). Filters were incubated with primary antibodies overnight at 4°C with shaking and then the membrane was probed with horseradish peroxidase-conjugated secondary antibody (1 : 5000 in TBST) for 1 h at RT, washed, and then visualized by ECL (Amersham Biosciences Inc., Piscataway, NJ, USA).

Cell cycle analysis

Cells were cultured under conditions described in the text and cell cycle analysis was determined by the propidium iodide method as described (Halloran and Fenton, 1998).

Isolation of CD138$^+$ myeloma cells

Mononuclear cells were isolated from bone marrow aspirates by Ficoll–Hypaque centrifugation, washed extensively, and incubated with anti-CD138-coated microbeads as per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). CD138$^+$ cells were purified by magnetic separation, washed, and then cultured under conditions described in the text. Purified MM cells were >95% positive for clonal kappa or lambda light chain.

Transfections and luciferase assays

ARP1 cells were cultured overnight in the absence of IL-6 and plated at 5×10^5 cells/0.5 ml. in 24-well plates. Cells were transfected using TransIT-LT1 lipid reagent (Mirus Corp., Madison, WI, USA) according to the manufacturer's recommendations. Each well received 2.5 μg of the luciferase expression vectors, and 0.25 μg of the pRL-TK vector encoding the HSV-thymidine kinase promoter upstream of the Renilla luciferase gene. Transfections were incubated for 6 h, followed by the addition of 0.5 ml of RPMI/10% FCS with or without IL-6 (final 5 ng/ml). Cells were cultured for an additional 18 h and assayed using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. Luminescence was determined using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Experiments were performed in triplicate and normalized to the level of Renilla luciferase expression. Data are presented as the mean $\pm$ the s.d. The vectors encoding 3.9 kb, 294 bp, or 140 bp of the human Mcl-1 promoter were kindly provided by Dr Steven W Edwards (University of Liverpool, UK).

Electrophoretic mobility shift assay (EMSA)

ARP1 cells were cultured overnight in the absence of IL-6. Cells (30×10^6) were then cultured in fresh RPMI/10% FCS with or without the addition of IL-6 (5 ng/ml) for an additional 30 min, and harvested for preparation of nuclear extracts. Cells were incubated in 500 μl cold hypotonic lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl), containing 1% NP40 for 30 min (all steps performed at 4°C). Nuclei were harvested by centrifugation, and resuspended in 60 μl of low salt buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25% glycerol) and 60 μl of high salt buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 25% glycerol) and incubated on ice for 30 min. After centrifugation at 15 000 r.p.m. for 15 min, the protein concentration was determined, and nuclear extracts were aliquoted and stored at -80°C. Sense and antisense hSIE (5′ GATC-CTT-CAT-TTC-CCG-TAA-ATC-CCT-A 3′) or FIRE (5′ GTC-CCC-CGG-CCG-GGG-AGG-CGC-T 3′) oligonucleotides containing 5′-GATC additions were annealed and labeled using Klenow polymerase. DNA–protein binding was performed for 20 min at RT in binding buffer (12 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 12% glycerol) containing 1 μg poly-dIdC and 5 μg of nuclear extracts. Unlabeled competitor oligonucleotides were added to a final concentration of 2 pmol. For supershift assays, an antibody directed against STAT3 (Cell Signaling Technology, Inc.) was added to the binding reaction for 20 min at RT. After the addition of loading dye, size fractionation was performed using nondenaturing 6% DNA retardation gels (Invitrogen-Novex, Carlsbad, CA, USA) in 0.5 $\times$ TBE. Gels were then dried and autoradiography was performed.

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References

Akgul C, Moulding DA, White MR and Edwards SW. (2000a). *FEBS Lett.,* **478,** 72–76.

Akgul C, Turner PC, White MR and Edwards SW. (2000b). *Cell. Mol. Life. Sci.,* **57,** 684–691.

Alcalay M, Orleth A, Sebastiani C, Meani N, Chiaradonna F, Casciari C, Sciurpi MT, Gelmetti V, Riganelli D, Minucci S, Fagioli M and Pelicci PG. (2001). *Oncogene*, **20,** 5680–5694.

Alessi DR, Cuenda A, Cohen P, Dudley DT and Saltiel AR. (1995). *J. Biol. Chem.,* **270,** 27489–27494.

Bergsagel PL, Chesi M, Nardini E, Brents LA, Kirby SL and Kuehl WM. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 13931–13936.

Bergsagel PL and Kuehl WM. (2001). *Oncogene*, **20,** 5611–5622.

Bright JJ, Du C and Sriram S. (1999). *J. Immunol.,* **162,** 6255–6262.

Calame KL. (2001). *Nat. Immunol.,* **2,** 1103–1108.

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS and Jove R. (1999). *Immunity,* **10,** 105–115.

Chao JR, Wang JM, Lee SF, Peng HW, Lin YH, Chou CH, Li JC, Huang HM, Chou CK, Kuo ML, Yen JJ and Yang-Yen HF. (1998). *Mol. Cell. Biol.,* **18,** 4883–4898.

Cory S and Adams JM. (2002). *Nat. Rev. Cancer.*, **2**, 647–656.

Craig RW. (2002). *Leukemia,* **16,** 444–454.

Derenne S, Monia B, Dean NM, Taylor JK, Rapp MJ, Harousseau JL, Bataille R and Amiot M. (2002). *Blood,* **100,** 194–199.

Di Cristofano A, Kotsi P, Peng YF, Cordon-Cardo C, Elkon KB and Pandolfi PP. (1999). *Science*, **285,** 2122–2125.

Epling-Burnette PK, Zhong B, Bai F, Jiang K, Bailey RD, Garcia R, Jove R, Djeu JY, Loughran Jr TP and Wei S. (2001). *J. Immunol.,* **166,** 7486–7495.

Fonseca R, Coignet LJ and Dewald GW. (1999). *Hematol. Oncol. Clin. North. Am.,* **13,** 1169–1180, viii.
Gauthier ER, Piche L, Lemieux G and Lemieux R. (1996). *Cancer Res.,* **56,** 1451–1456.
Ge NL and Rudikoff S. (2000). *Oncogene,* **19,** 4091–4095.
Gojo I, Zhang B and Fenton RG. (2002). *Clin. Cancer Res.,* **8,** 3527–3538.
Goldstein JC, Waterhouse NJ, Juin P, Evan GI and Green DR. (2000). *Nat. Cell. Biol.*, **2,** 156–162.
Gross A, McDonnell JM and Korsmeyer SJ. (1999). *Genes Dev.,* **13,** 1899–1911.
Hallek M, Bergsagel PL and Anderson KC. (1998). *Blood,* **91,** 3–21.
Halloran PJ and Fenton RG. (1998). *Cancer Res.,* **58,** 3855–3865.
Hanada M, Delia D, Aiello A, Stadtmauer E and Reed JC. (1993). *Blood,* **82,** 1820–1828.
Helgason CD, Damen JE, Rosten P, Grewal R, Sorensen P, Chappel SM, Borowski A, Jirik F, Krystal G and Humphries RK. (1998). *Genes Dev.*, **12,** 1610–1620.
Hideshima T, Nakamura N, Chauhan D and Anderson KC. (2001). *Oncogene,* **20,** 5991–6000.
Huang DC and Strasser A. (2000). *Cell,* **103,** 839–842.
Huang HM, Huang CJ and Yen JJ. (2000). *Blood,* **96,** 1764–1771.
Hyun T, Yam A, Pece S, Xie X, Zhang J, Miki T, Gutkind JS and Li W. (2000). *Blood,* **96,** 3560–3568.
Jelinek DF, Witzig TE and Arendt BK. (1997). *J. Immunol.,* **159,** 487–496.
Johnstone RW, Ruefli AA and Lowe SW. (2002). *Cell*, **108,** 153–164.
Jourdan M, De Vos J, Mechti N and Klein B. (2000). *Cell Death Differ.,* **7,** 1244–1252.
Kaufmann SH, Karp JE, Svingen PA, Krajewski S, Burke PJ, Gore SD and Reed JC. (1998). *Blood,* **91,** 991–1000.
Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S, Wang HG, Zhang X, Bullrich F, Croce CM, Rai K, Hines J and Reed JC. (1998). *Blood,* **91,** 3379–3389.
Kitada S, Zapata JM, Andreeff M and Reed JC. (2000). *Blood,* **96,** 393–397.
Kozopas KM, Yang T, Buchan HL, Zhou P and Craig RW. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 3516–3520.
Liu H, Perlman H, Pagliari LJ and Pope RM. (2001). *J. Exp. Med.,* **194,** 113–126.
Martinou JC and Green DR. (2001). *Nat. Rev. Mol. Cell. Biol.*, **2,** 63–67.
McConkey DJ, Chandra J, Wright S, Plunkett W, McDonnell TJ, Reed JC and Keating M. (1996). *J. Immunol.,* **156,** 2624–2630.
McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP and Korsmeyer SJ. (1989). *Cell,* **57,** 79–88.
Metcalf D. (1998). *Stem Cells,* **16,** 3–11.
Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, Leeder JS, Freedman M, Cohen A, Gazit A, Levitzki A and Roifman CM. (1996). *Nature,* **379,** 645–648.
Miguel-Garcia A, Orero T, Matutes E, Carbonell F, Miguel-Sosa A, Linares M, Tarin F, Herrera M, Garcia-Talavera J and Carbonell-Ramon F. (1998). *Haematologica,* **83,** 298–304.
Moulding DA, Giles RV, Spiller DG, White MR, Tidd DM and Edwards SW. (2000). *Blood,* **96,** 1756–1763.
Ong F, van Nieuwkoop JA, de Groot-Swings GM, Hermans J, Harvey MS, Kluin PM and Kluin-Nelemans JC. (1995). *Leukemia,* **9,** 1282–1284.
Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S and Pouyssegur J. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 8319–8323.
Pepper C, Bentley P and Hoy T. (1996). *Br. J. Haematol.,* **95,** 513–517.
Puthier D, Bataille R and Amiot M. (1999a). *Eur. J. Immunol.,* **29,** 3945–3950.
Puthier D, Derenne S, Barille S, Moreau P, Harousseau JL, Bataille R and Amiot M. (1999b). *Br. J. Haematol.,* **107,** 392–395.
Puthier D, Pellat-Deceunynck C, Barille S, Robillard N, Rapp MJ, Juge-Morineau N, Harousseau JL, Bataille R and Amiot M. (1999c). *Leukemia*, **13,** 289–294.
Raff MC. (1992). *Nature*, **356,** 397–400.
Raghoebier S, van Krieken JH, Kluin-Nelemans JC, Gillis A, van Ommen GJ, Ginsberg AM, Raffeld M and Kluin PM. (1991). *Blood*, **77,** 1560–1564.
Rajkumar SV, Greipp PR. (1999). *Hematol. Oncol. Clin. North Am.*, **13,** 1295–1314, xi.
Reed JC. (1995). *Hematol, Oncol, Clin, North. Am.,* **9,** 451–473.
Reed JC. (1998). *Oncogene*, **17,** 3225–3236.
Rowley JD. (1988). *J. Clin. Oncol.*, **6,** 919–925.
Schimmer AD, Hedley DW, Penn LZ and Minden MD. (2001). *Blood,* **98,** 3541–3553.
Schubert KM and Duronio V. (2001). *Biochem. J.*, **356,** 473–480.
Slifka MK, Antia R, Whitmire JK and Ahmed R. (1998). *Immunity*, **8,** 363–372.
Sordet O, Bettaieb A, Bruey JM, Eymin B, Droin N, Ivarsson M, Garrido C and Solary E. (1999). *Cell Death Differ.,* **6,** 351–361.
Thomas A, El Rouby S, Reed JC, Krajewski S, Silber R, Potmesil M and Newcomb EW. (1996). *Oncogene,* **12,** 1055–1062.
Townsend KJ, Zhou P, Qian L, Bieszczad CK, Lowrey CH, Yen A and Craig RW. (1999). *J. Biol. Chem.,* **274,** 1801–1813.
Tricot G, Sawyer JR, Jagannath S, Desikan KR, Siegel D, Naucke S, Mattox S, Bracy D, Munshi N and Barlogie B. (1997). *J. Clin. Oncol.,* **15,** 2659–2666.
Tu Y, Renner S, Xu F, Fleishman A, Taylor J, Weisz J, Vescio R, Rettig M, Berenson J, Krajewski S, Reed JC and Lichtenstein A. (1998). *Cancer Res.,* **58,** 256–262.
Zhan F, Hardin J, Kordsmeier B, Bumm K, Zheng M, Tian E, Sanderson R, Yang Y, Wilson C, Zangari M, Anaissie E, Morris C, Muwalla F, van Rhee F, Fassas A, Crowley J, Tricot G, Barlogie B and Shaughnessy Jr J. (2002). *Blood*, **99,** 1745–1757.
Zhang B and Fenton RG. (2002). *J. Cell Physiol.,* **193,** 42–54.
Zhang B, Gojo I and Fenton RG. (2002). *Blood,* **99,** 1885–1893.
Zhou P, Levy NB, Xie H, Qian L, Lee CY, Gascoyne RD and Craig RW. (2001). *Blood,* **97,** 3902–3909.