Cytoplasmic domains of the common β -chain of the GM-CSF/IL-3/IL-5 receptors that are required for inducing differentiation or clonal suppression in myeloid leukaemic cell lines

Alison Smith, Donald Metcalf and Nicos A. Nicola¹

The Walter and Eliza Hall Institute of Medical Research and
The Cooperative Research Centre for Cellular Growth Factors,
PO Royal Melbourne Hospital, 3050 Victoria, Australia

¹Corresponding author

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine that controls the production and function of myeloid cells by interaction with a cell surface receptor composed of a specific ligand-binding α -chain (hGMR α) and a shared signal-transducing β -chain (β c). Co-expression of human GMR α -chain and wild-type human β c in two murine leukaemic cell lines (M1 and WEHI-3B D+) conferred the ability to terminally differentiate into macrophages when stimulated with human GM-CSF. Analysis of cytoplasmic truncation mutants of β c showed that residues to amino acid 783 (numbering from the first amino acid of the leader sequence) were sufficient for the GM-CSF-dependent induction of all aspects of differentiation in both cell types. However, shorter truncations selectively lost, in a cell-specific manner, first the capacity to induce macrophage migration in agar and then cell surface differentiation antigens and clonal suppression of proliferative potential. The data suggest that different aspects of the differentiated phenotype can be dissociated with the required signalling pathways originating from distinct regions of the receptor cytoplasmic domain and cooperating to produce a fully differentiated macrophage. The cooperativity of these pathways and limiting cell signalling intermediate pool sizes could explain the observed cell line differences and may have implications for normal haemopoiesis.
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Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that regulates the survival, proliferation and differentiation of progenitor cells of neutrophilic and eosinophilic granulocytes, macrophages and megakaryocytic cells. In addition, it stimulates the functional activity of mature granulocytes and macrophages (Metcalf and Nicola, 1995). GM-CSF has found clinical utility in enhancing the recovery of haematopoietic cells following the treatment of cancer patients with chemotherapy (Grant and Heel, 1992). Recent studies in mice engineered to lack functional expression of the GM-CSF gene have suggested that it plays only a minor role in steady-state haematopoiesis but is essential for normal macrophage

function, at least in the lung, and for mounting a protective response to certain infections (Dranoff *et al.*, 1994; Stanley *et al.*, 1994).

GM-CSF exerts its actions through binding to cell surface receptors that consist of at least two different subunits. The α -chain (hGM-CSFR α) binds GM-CSF specifically but with relatively low affinity (Gearing *et al.*, 1989a). Subsequent interaction with a β -chain (β c) generates a high-affinity receptor complex capable of proliferative signal transduction (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991; Park *et al.*, 1992; Sakamaki *et al.*, 1992). The β -subunit is shared by the functional receptors for interleukin-3 (IL-3) and interleukin-5 (IL-5) but, when expressed alone, β c shows little binding affinity for either GM-CSF, IL-3 or IL-5 (Tavernier *et al.*, 1991; Hara and Miyajima, 1992).

Reconstitution experiments have shown that the cytoplasmic domains of both the GM-CSFR α and β c are required to transduce a proliferative signal in CTLL-2 cells (Sakamaki *et al.*, 1992). However, chimeric receptors in which the β c cytoplasmic domain was connected to the extracellular domain of the GM-CSF or IL-5 receptor α -subunits were still able to signal in a ligand-dependent manner in FDC-P1 or Ba/F3 cells, respectively (Takaki *et al.*, 1993; Eder *et al.*, 1994). It was suggested that these chimeric receptors formed dimers of the β -chain cytoplasmic domains (possibly through heterodimerization of the chimeric receptor with endogenous mouse β -chains). Since, in these systems, the cytoplasmic domain of the α -chain was not required for proliferative signalling, it was further suggested that its primary role in this situation was simply to promote or stabilize dimerization of the two β -chains. This conclusion is supported by the observation that certain mutations in β c lead to constitutive proliferative signalling, possibly by ligand-independent dimerization of the β -chains (Jenkins *et al.*, 1995).

Deletion analysis of the cytoplasmic domain of β c has shown that the membrane-proximal region of ~32 amino acids is sufficient to stimulate a transient mitogenic response in factor-dependent cell lines (Sakamaki *et al.*, 1992; Sato *et al.*, 1993; Kinoshita *et al.*, 1995). This region contains a proline-rich sequence, termed box-1, that is relatively conserved among several cytokine receptors and serves as a binding site for the cytoplasmic tyrosine kinase JAK2 (Quelle *et al.*, 1994; Zhao *et al.*, 1995). JAK2 is apparently constitutively associated with β c through its own N-terminal domain but its kinase activity is activated only after GM-CSF binding (Zhao *et al.*, 1995). The box-1 region is also required for the induction of *c-myc* mRNA expression which is associated with competence for cell proliferation (Sakamaki *et al.*, 1992; Sato *et al.*, 1993). A second conserved cytokine receptor element (box-2) occurs ~60 amino acids C-terminal to the transmembrane domain and enhances

Fig. 1. The structure of wild-type β_c (boxed) is depicted showing the extracellular domain, consisting of two subdomains with the defining four cysteine residues (C) and the WSXWS motif (black). The intracellular domain of wild-type β_c and truncations are shown above. TM, transmembrane domain. Box 1 and Box 2 homology domains are shaded. The number associated with each truncation indicates the amino acid at which the receptor was truncated with position 1 being the first amino acid of the leader sequence. 17 amino acids (the leader sequence) should be deducted from this numbering system to equate mutants with those used by Sakamaki *et al.* (1992), Sato *et al.* (1993) and Kinoshita *et al.* (1995). A summary of the data follows showing the ability of each truncation to promote the proliferation of CTLL-2 cells, differentiation in agar (dispersed colonies) (D), clonal suppression (C.S.) or up-regulation of surface antigens in response to hGM-CSF in M1 or WEHI-3B D⁺ cells. ND, not determined.

the proliferative response induced by the box-1 element (Sakamaki *et al.*, 1992).

Cells expressing mutant β_c -chains with truncated cytoplasmic regions limited to either box-1 alone or box-1 and box-2 exhibited little tyrosine phosphorylation within the cells when stimulated by GM-CSF (Sakamaki *et al.*, 1992). Several cellular targets of GM-CSF-induced tyrosine phosphorylation have been identified and include the β_c subunit itself, *shc*, *raf*, MAP kinase, *vav*, *fps*, JAK2 and STAT5A and STAT5B (Miyajima *et al.*, 1993; Mui *et al.*, 1995). Deletion analysis of the cytoplasmic domain of β_c has indicated that box-1, as well as elements downstream of box-1 and box-2, are required for many of these phosphorylation events including the activation of the *shc*-*sos*-*ras*-*raf*-MAP kinase pathway involved in transcriptional activation of genes like *c-fos* (Sakamaki *et al.*, 1992; Sato *et al.*, 1993; Itoh *et al.*, 1996). These data suggest that this pathway may not be essential for GM-CSF-induced cellular proliferation.

Despite the mapping of particular biochemical signalling pathways to specific regions of the cytoplasmic domain of β_c , the correlation of these pathways with different types of biological responses is less well understood. The membrane-proximal domain has been associated with cell proliferation but whether this is mediated by the JAK-STAT pathway or by other receptor-associated cytoplasmic kinases such as *Lyn*, *Fyn* and *Fes* or by as yet undetermined pathways is not known. Recent evidence has suggested that the C-terminal domain of β_c , associated with activation of the Ras/MAPK pathway, may also be involved in suppression of apoptotic death (Kinoshita *et al.*, 1995). However, there is currently very little information on the receptor domains of β_c and signalling pathways required for the induction of terminal differentiation pathways to

granulocytes or macrophages. In the present study it is shown that functional expression of hGM-CSFR α and β_c in murine M1 and WEHI-3B D⁺ myeloid leukaemic cell lines confers on them the capacity to respond to hGM-CSF by differentiating to macrophages and becoming clonally suppressed. β_c cytoplasmic deletion mutants were used to map the receptor domains that mediate these processes in leukaemic cells.

Results

Expression of wild-type and mutant β -chain cDNAs

To examine functional regions of the human β_c chain necessary for transducing hGM-CSF induced signals, a series of mutant human β_c cDNAs was constructed with sequential deletions of the cytoplasmic domain (Figure 1). The notation for each truncation represents the number of amino acids from the N-terminus including the leader sequence. Thus, our mutant numbering system is 17 amino acids longer than that used by Sakamaki *et al.* (1992), Sato *et al.* (1993) and Kinoshita *et al.* (1995) which excluded the presumptive leader sequence. Both wild-type and mutant β_c cDNAs were co-expressed with the hGMR α in M1, WEHI-3B D⁺ and CTLL-2 cells and transfected cell lines were analysed for receptor expression by performing saturation binding experiments with [¹²⁵I]hGM-CSF. Scatchard transformation of the data (Figure 2) showed that M1 cell lines expressing hGMR α alone displayed a single class of low-affinity receptors with an apparent equilibrium dissociation constant (K_D) of ~2.2 nM with an average of 25 000 receptors per cell (Table I). Parental cell lines did not bind human GM-CSF to any significant level, nor did cell lines transfected with empty vector or cell lines expressing β_c alone (data not shown).

Cell lines transfected with both hGMRα and wild-type βc displayed two classes of receptor, a low-affinity component comparable with that detected on cells expressing the hGMRα alone, and a high-affinity component with a K_D of ~55 pM (Figure 2 and Table I). Cells

Fig. 2. Scatchard analysis of 125 I-labelled human GM-CSF binding to M1 parental cells, M1 cells transfected with hGMR α-chain alone and M1 cells transfected with hGMR α-chain and wild-type or truncated GMR β-chains. Cells were incubated with decreasing concentrations of labelled hGM-CSF in the presence or absence of 10- to 100-fold excess of unlabelled hGM-CSF on ice for 3 h. Bound and free hGM-CSF were separated by centrifugation through FCS. The bound and free fractions were quantitated in a gamma-counter. Scatchard transformation of the data was performed assuming 100% bindability of labelled ligand.

expressing hGMRα and the various truncated βcs also displayed both high- and low-affinity receptor sites with K_D values and receptor numbers comparable with that for wild-type βc (Table I). In each case the number of high-affinity receptors was 10- to 100-fold lower than the number of low-affinity sites, indicating that the number of hGM-CSFRα-chains expressed was in excess. Essentially the same data were obtained for expression of α- and β-chains in CTLL-2 and WEHI-3B D+ cells (Table I). However, although we were able to generate both CTLL-2 and M1 cells expressing the β487 mutant, we were not able to detect any statistically significant high-affinity receptor sites on WEHI-3B D+ cells transfected with this mutant cDNA. Several transfections with the β487 cDNA were performed resulting in the production of over 40 clones but none displayed a statistically significant level of high-affinity binding. The reason for this discrepancy is unknown, since the same cDNA was used for transfection into all three cell lines.

In all of the following results, only cell clones that displayed statistically significant two-site binding were selected for analysis since these verified expression of the transfected βc mutant cDNA. Moreover, since some cell clones displayed variation in behaviour in the various assays used, data are only presented if they were typical of the majority of such clones and if they were replicated using at least two or three independently derived cell clones.

Function of βc and cytoplasmic deletion mutants in CTLL-2 proliferative signal transduction

It has been previously established that hGM-CSF is capable of stimulating the proliferation of mouse Ba/F3 and CTLL-2 cells co-expressing hGMRα and βc (Sakamaki *et al.*, 1992; Sato *et al.*, 1993; Kinoshita *et al.*, 1995), indicating that murine signalling molecules can

Table I. Binding affinities and number of hGM-CSF receptors on cell transfectants^a

Parental cell line	Transfectant	Low affinity		High affinity	
		K_D (nM)	Receptors/cell ($\times 10^{-4}$)	K_D (pM)	Receptors/cell ($\times 10^{-3}$)
CTLL-2	α	1.8 ± 0.4	2.8 ± 1.6	—	—
	αβwt	7.2 ± 3.9	5.2 ± 3.0	107 ± 56	6.9 ± 3.5
	αβ783	5.8 ± 5.0	5.0 ± 2.1	27 ± 26	3.1 ± 2.6
	αβ626	14 ± 7	10 ± 8	153 ± 76	6.8 ± 3.2
	αβ541	2.6 ± 0.3	3.1 ± 1.2	48 ± 27	7.1 ± 4.9
	αβ487	4.1 ± 1.4	0.7 ± 0.2	80 ± 51	0.3 ± 0.1
	αβ461	33 ± 18	1.9 ± 0.8	71 ± 38	0.3 ± 0.04
	α	3.7 ± 1.2	2.3 ± 1.2	—	—
WEHI-3B D+	αβwt	4.2 ± 0.8	2.8 ± 0.7	40 ± 28	0.7 ± 0.6
	αβ783	11 ± 8	14 ± 9	15 ± 10	0.7 ± 0.3
	αβ626	9.3 ± 3.3	5.4 ± 1.9	91 ± 49	1.6 ± 0.9
	αβ541	7.0 ± 1.3	7.9 ± 1.2	24 ± 8	0.9 ± 0.2
	αβ487	n.d.	n.d.	n.d.	n.d.
	αβ461	5.5 ± 1.3	4.5 ± 1.7	80 ± 10	1.9 ± 0.6
	α	2.2 ± 0.2	2.5 ± 3.4	—	—
	αβwt	3.1 ± 1.1	1.6 ± 0.4	55 ± 26	2.7 ± 1.4
M1	αβ783	1.2 ± 2.0	1.5 ± 0.1	189 ± 19	4.9 ± 1.3
	αβ626	61 ± 46	3.4 ± 1.2	256 ± 102	5.0 ± 1.6
	αβ541	13 ± 3	2.5 ± 2.0	109 ± 55	6.4 ± 2.0
	αβ487	16 ± 12	1.5 ± 0.7	27 ± 50	2.5 ± 0.7
	αβ461	12 ± 7	0.7 ± 0.2	136 ± 67	5.0 ± 2.5
	α	—	—	—	—
	αβwt	—	—	—	—
	αβ783	—	—	—	—

^aAnalysis was performed on two to six individual clones for each cell line. All two-site binding profiles, where indicated, were statistically significant ($P < 0.05$) better fits to the data than one-site fits. n.d., not determined.

Fig. 3. Factor-dependent proliferation of parental CTLL-2 cells and CTLL-2 transfectants stimulated with mIL-2 (●) or hGM-CSF (○). Cells were incubated at 200 cells/well in a volume of 15 μ l with varying concentrations of IL-2 or GM-CSF. The number of viable cells was assessed microscopically after a 48–72 h incubation period.

successfully interact with the human receptor chains. Short-term proliferation in Ba/F3 cells minimally required the box-1 homology domain located in the membrane-proximal region of β c. The more distally located box-2 was not critical for growth signal transduction but enhanced the proliferative response of Ba/F3 cells to hGM-CSF. It was of interest to determine if the region of β c required for cell proliferation could be separated from the region required for differentiation using the same set of mutants. Mouse CTLL-2 cells were chosen to examine growth signalling as they lack endogenous receptors for GM-CSF, IL-3 and IL-5, including β c. Thus, any response detected to human GM-CSF could only be due to the presence of a functional human β c.

The proliferation of CTLL-2 transfectants in response to hGM-CSF stimulation (measured as an increase in cell numbers in microwell assays after 48–72 h) was found to require expression of both hGMR α and β c (Figure 3). Half-maximal stimulation of $\alpha\beta$ c transfectants occurred at a human GM-CSF concentration between 40 and 100 pg/ml. CTLL-2 cells expressing hGMR α and either β 783 or β 626 were also capable of proliferating in response to hGM-CSF at a consistently lower half-maximal concentration (between 5 and 15 pg/ml with at least three independent clones of each). However, CTLL-2 $\alpha\beta$ 541, $\alpha\beta$ 487 or $\alpha\beta$ 461 transfectants were unable to proliferate in response to hGM-CSF, even at concentrations up to 10 μ g/ml. In each case, several clones expressing both high- and low-affinity receptor sites were tested, and although the response of these cells to IL-2 was comparable with that of the parental cell line, they were all equally unresponsive to hGM-CSF. Therefore, it appears that the critical membrane-proximal region required for hGM-CSF-induced proliferation in mouse CTLL-2 cells has a

Fig. 4. Differentiation of M1 cells in response to hGM-CSF. Parental M1 cells, M1 cells expressing GMR α -chain, or M1 cells expressing hGMR α -chain and wild-type β c were cultured at 200 cells/ml in semi-solid agar with various concentrations of hGM-CSF as indicated. The proportion of colonies containing differentiated cells (dispersed colonies) was determined after 7 days' incubation at 37°C. The left-hand panels show the percentage of colonies displaying a differentiated phenotype; the right-hand panels show the total number of colonies present. ○, ● represent two independent clones; the bars on the right of each panel indicate the ability of these cells to respond to a maximal concentration of LIF.

C-terminal border that lies between amino acids 541 and 626.

GM-CSF induces macrophage differentiation in M1 and WEHI-3B D+ cells expressing hGMR α and β c

Differentiation of murine leukaemic M1 cells in response to LIF is characterized by a decrease in proliferative capacity (reduced colony numbers) and distinct morphological changes, such as an increase in cell size and granularity and the increased expression of macrophage-specific surface markers (Metcalf *et al.*, 1988; Lord *et al.*, 1991). In semi-solid agar cultures, unstimulated M1 cells form tightly packed colonies and have an immature blast-like morphology. In contrast, in LIF-stimulated cultures, dispersed colonies form as the differentiated macrophages migrate away from the colony. Associated with the appearance of dispersed colonies is a significant decrease in total colony numbers and size as the cells cease to proliferate.

Parental M1 cells and M1 cells expressing either hGMR α or β c alone exhibited a normal response to LIF, but were unable to differentiate in response to hGM-CSF (Figure 4 and data not shown). In contrast, M1 cells expressing hGMR α and β c responded normally to LIF, but also responded to hGM-CSF in dramatic fashion with a half-maximal response at $\sim$ 0.05 ng/ml. In this response, there was a marked reduction in colony numbers and

Fig. 5. Differentiation of WEHI-3B D+ cells in response to hGM-CSF. Parental WEHI-3B D+ cells, WEHI-3B D+ cells expressing hGMR α -chain alone, or WEHI-3B D+ cells expressing hGMR α -chain and wild-type β c were cultured at 100 cells/ml semi-solid agar with various concentrations of hGM-CSF as indicated. The proportion of colonies containing differentiated cells was determined after 7 days' incubation at 37°C. The left-hand panels show the percentage of colonies displaying a differentiated phenotype; the right-hand panels show the total number of colonies present. ●, ○ represent two independent clones; the bars on the right of each panel indicate the ability of these cells to respond to a maximal concentration of G-CSF.

size with full dispersion of the few cells comprising the colonies.

Mouse leukaemic WEHI-3B D+ cells stimulated to differentiate by G-CSF displayed a weaker response than LIF-stimulated M1 cells in that most differentiated colonies were merely composed of a tight central core of cells surrounded by a halo of migrating cells. Usually there was no significant suppression of colony numbers and sometimes even a slight increase in colony numbers and size, in sharp contrast to the action of LIF on M1 cells. As expected, the parental WEHI-3B D+ cell line was completely unresponsive to hGM-CSF (Figure 5).

WEHI-3B D+ cells expressing hGMR α alone responded to G-CSF in a similar manner to cells of the parental line but were able to form some differentiated colonies with the highest concentrations of hGM-CSF (Figure 5). WEHI-3B D+ cells express high-affinity murine receptors for IL-3 and GM-CSF that include endogenous mouse β c. The present results suggest that, at high concentrations of hGM-CSF, the hGMR α is able to interact with murine β c to induce some differentiation. This agrees with the previous demonstration that, although the interaction between hGMR α and mGMR β c is one of low affinity, the hGMR α /m β c complex is capable of delivering a proliferative signal in response to high concentrations of hGM-CSF (Metcalf *et al.*, 1990; Kitamura *et al.*, 1991) and extends the finding to differentiative signalling.

WEHI-3B D+ cells expressing both hGMR α and β c

responded normally to G-CSF, but were also able to respond to hGM-CSF. The hGM-CSF response, however, was much stronger than the response to G-CSF and more comparable with the response seen in M1 cells stimulated with either LIF or hGM-CSF. In particular, morphological differentiation to macrophages was more evident with hGM-CSF (Figure 6) and, in contrast to the response to G-CSF, there was a profound clonal suppression induced by hGM-CSF. The number of high-affinity hGM-CSF receptors expressed on these cells was on average 700 per cell, which is ~10-fold higher than the number of G-CSF receptors per cell. These results may suggest that the number of functional receptors able to interact with a ligand is a critical factor in determining the quantitative response of a cell to a given stimulus.

To further assess the extent of macrophage differentiation stimulated by hGM-CSF in both M1 and WEHI-3B D+ cells, liquid suspension cultures were performed and the cells harvested after 4 days to assess their morphology microscopically (Figure 6). M1 cells expressing hGMR α and β c showed clear morphological differentiation of blast cells into macrophages in response to hGM-CSF, similar to the response of M1 cells to LIF (Figure 6, panels A–C). The differentiated cells displayed a larger cytoplasm/nuclear ratio, eccentric nucleus and foamy cytoplasm. A similar response to hGM-CSF was seen with WEHI-3B D+ cells expressing hGMR α and β c and this was much more striking than the weak morphological differentiation seen with G-CSF (Figure 6, panels D–F). Clear morphological differentiation was also observed with M1 and WEHI-3B D+ cells expressing hGMR α and β 783 or β 626 when stimulated by hGM-CSF (Figure 6, panels G–I). This was often accompanied by extensive cell adherence to the tissue culture plates which usually exceeded that seen even for cells expressing full-length β c. M1 cells expressing forms of β c shorter than 626 did not show morphological differentiation in response to hGM-CSF, whereas in WEHI-3B D+ cells the β 541, but not β 461, truncation retained the capacity to induce morphological differentiation (data not shown).

GM-CSF increases the expression of macrophage-specific surface markers in M1 and WEHI-3B D+ cells expressing hGMR α and β c

We examined the expression of surface markers known to be associated with macrophage differentiation to further characterize the maturation changes stimulated by hGM-CSF in both M1 and WEHI-3B D+ cells.

When parental M1 cells were induced to differentiate into macrophages by LIF, the expression of several cell surface markers was increased. These included Fc γ RII or III, Mac-1, the receptor for macrophage-stimulating factor, *c-fms*, F4/80 and Gr-1. After 4 days of stimulation, the level of expression as measured by FACS analysis, was generally 10- to 13-fold higher than in control cultures (Table II). LIF-induced expression of Mac-1 was generally not as strong (4- to 5-fold). An average fold induction of <2.0 was not considered to be significantly different from the background induction seen for isotype control antibodies for both M1 and WEHI-3B D+ stimulated with either LIF or G-CSF.

M1 cells expressing hGMR α and β c were stimulated with hGM-CSF for 4 days and then analysed for surface

Fig. 6. Morphology of M1 and WEHI-3B D+ cells containing hGMR α and β c in liquid suspension cultures. All stimuli were used at 10^3 U/ml. (A) M1 $\alpha\beta$ wt cells stimulated by saline for 4 days; (B) M1 $\alpha\beta$ wt cells stimulated by LIF for 4 days; (C) M1 $\alpha\beta$ wt cells stimulated by hGM-CSF for 4 days; (D) WEHI-3B D+ $\alpha\beta$ wt cells stimulated by saline for 4 days; (E) WEHI-3B D+ $\alpha\beta$ wt cells stimulated by G-CSF for 4 days; (F) WEHI-3B D+ $\alpha\beta$ wt cells stimulated by hGM-CSF for 4 days; (G) M1 $\alpha\beta$ 626 cells stimulated by saline for 4 days; (H) M1 $\alpha\beta$ 626 cells stimulated by hGM-CSF for 4 days; (I) WEHI-3B D+ $\alpha\beta$ 626 cells stimulated by hGM-CSF for 4 days.

Table II. Average fold increase over saline controls of the expression of granulocyte and macrophage-specific surface antigens in M1 cells stimulated with mLIF or hGM-CSF

Cell line	Stimulus	Fc γ R	Mac-1	<i>c-fms</i>	F4/80	Gr-1
M1 p	mLIF	13.4	3.8	11.4	10.1	12.8
	hGM-CSF	1.2	0.7	1.0	0.9	0.8
M1 α	mLIF	11.0	7.2	10.6	9.2	6.2
	hGM-CSF	1.4	0.8	1.3	0.9	1.0
M1 $\alpha\beta$ wt	mLIF	13.3	7.4	11.3	4.8	4.2
	hGM-CSF	5.9	7.6	5.3	12.2	4.9
M1 $\alpha\beta$ 783	mLIF	12.3	9.2	12.4	4.2	2.7
	hGM-CSF	12.2	14.5	14.1	11.9	8.4
M1 $\alpha\beta$ 626	mLIF	12.4	8.9	12.8	7.8	2.7
	hGM-CSF	6.6	10.6	8.8	20.6	2.2
M1 $\alpha\beta$ 541	mLIF	17.1	10.0	13.3	8.6	8.9
	hGM-CSF	1.3	1.0	1.2	1.1	1.3
M1 $\alpha\beta$ 487	mLIF	14.3	16.8	12.6	5.6	12.1
	hGM-CSF	0.9	1.1	1.1	0.9	1.2
M1 $\alpha\beta$ 461	mLIF	24.0	6.7	30.4	24.3	19.5
	hGM-CSF	1.2	1.0	0.9	1.0	0.9

marker expression. The expression of Fc γ R, Mac-1, *c-fms*, F4/80 and Gr-1 was found to be elevated compared with the saline controls (Figure 7). The level of induction for Fc γ R and *c-fms* was generally lower than that stimulated by LIF (Table II), but interestingly, hGM-CSF was able to induce the expression of F4/80 to ~3-fold the level of induction achieved by LIF stimulation. The level of Mac-1

and Gr-1 expression induced by hGM-CSF was similar to that induced by LIF.

The weak differentiative response of WEHI-3B D+ cells to G-CSF as noted in agar cultures was paralleled by the minimal induction of surface markers after 4 days of stimulation of cells in suspension cultures. G-CSF did not significantly induce the increased expression of Fc γ R,

Fig. 7. Up-regulation of myeloid surface antigens on M1 and WEHI-3B D+ cells expressing hGMR α-chain and wild-type βc. Cells were cultured in the presence of 10³ U/ml hGM-CSF (solid line) or saline (dashed line) for 4 days at 37°C. Cell surface expression of the various antigens was analysed by flow cytometry as described in Materials and methods.

Table III. Average fold increase over saline controls of expression of granulocyte and macrophage-specific surface antigens in WEHI-3B D+ cells stimulated with mG-CSF or hGM-CSF

Cell line	Stimulus	FcγR	Mac-1	<i>c-fms</i>	F4/80	Gr-1
D+ p	mG-CSF	1.9	2.1	2.0	1.6	2.7
	hGM-CSF	0.8	1.1	1.0	1.0	1.1
D+ α	mG-CSF	1.5	1.8	1.5	2.0	2.2
	hGM-CSF	0.9	0.9	1.0	1.0	1.1
D+ αβwt	mG-CSF	1.4	1.7	1.6	1.8	1.9
	hGM-CSF	1.4	4.8	1.5	8.0	2.6
D+ αβ783	mG-CSF	1.4	1.3	1.6	2.2	2.3
	hGM-CSF	1.4	2.8	0.9	5.6	3.1
D+ αβ626	mG-CSF	1.6	1.2	1.8	1.4	2.1
	hGM-CSF	1.6	2.6	1.4	2.2	2.1
D+ αβ541	mG-CSF	1.4	2.0	1.6	1.4	2.2
	hGM-CSF	0.9	3.3	1.5	10.7	8.3
D+ αβ461	mG-CSF	1.4	2.0	1.5	1.5	4.7
	hGM-CSF	1.0	1.0	0.9	1.0	1.5

Mac-1, *c-fms* or F4/80 (Table III). It should be noted, however, that the basal level of expression of these markers on WEHI-3B D+ cells was as least as high as that achieved with M1 cells after stimulation (Figure 7), suggesting that, in control cultures, these cells may already be more differentiated towards macrophages than are M1 cells. Interestingly, G-CSF did induce a consistent 2- to 4-fold increase in the expression of the granulocytic surface marker Gr-1.

The induction of FcγR and *c-fms* by hGM-CSF on WEHI-3B D+ cells expressing hGMRα and βc was as poor as that induced by G-CSF (Figure 7). However, hGM-CSF was able to induce a significant increase in the expression of Mac-1, F4/80 and Gr-1 (Figure 7 and Table III).

Effects of βc truncation mutants on hGM-CSF-induced differentiation and clonal suppression of M1 and WEHI-3B D+ cells

In all respects, the β783 truncated mutant, in which the C-terminal 114 amino acids were deleted, was as effective as wild-type βc in the induction of differentiation and in clonal suppression of M1 and WEHI-3B D+ cells by human GM-CSF. This included the induction of dispersed colonies and a reduction in colony numbers in agar

(Figures 8 and 9), as well as the induction of morphological differentiation and induction of the macrophage surface markers Fcγ receptor, Mac-1, *c-fms*, F4/80 and Gr-1 in M1 cells and Mac-1, F4/80 and Gr-1 in WEHI-3B D+ cells (Tables II and III).

The truncation mutant β626 displayed intermediate behaviour. In M1 cells this mutant permitted a nearly complete differentiation response to hGM-CSF with respect to dispersed colonies in agar, clonal suppression and induction of surface markers although the ability to form dispersed colonies in agar required 50- to 100-fold higher concentrations of hGM-CSF than did M1 cells expressing the longer forms of βc (Figure 8). In cultures of WEHI-3B D+ β626 cells, dispersed colonies did not develop in response to hGM-CSF but both morphological differentiation to macrophages and an induction of the cell surface markers Mac-1, F4/80 and Gr-1 was observed (Figures 6 and 9 and Tables II and III). In both transfected cell lines, strong clonal suppression was seen in response to hGM-CSF (Figures 8 and 9). Despite the weakness of differentiation induction in WEHI-3B D+ cells as assessed by colony morphology, at least some cells from both types of cell line transfected with β626 showed a striking hGM-CSF-induced morphological transformation to adherent macrophages with extended cell processes. Indeed, the

Fig. 8. Differentiation of M1 cells expressing hGMR α -chain and mutant β c in response to hGM-CSF. M1 cells expressing hGMR α -chain and either β 783, β 626, β 541, β 487 or β 461 were cultured as described previously with various concentrations of hGM-CSF. Cultures were scored for percentage of differentiated colonies at day 7. The left-hand panels show the percentage of colonies containing differentiated cells; the right-hand panels show the total number of colonies per plate. $\circ$, $\bullet$ indicate the responses of two independent clones; the bars to the right of each panel indicate the ability of the cells to respond to a maximal concentration of LIF.

appearance of these adherent cells was often more dramatic than with cells from β 783 or wild-type β c clones.

Even with the highest concentrations of hGM-CSF, the truncation mutants β 541, β 487 and β 461 expressed along with hGMR α in M1 cells were ineffective in the induction of clonal suppression, the formation of dispersed colonies in agar or the induction of macrophage surface markers. (Figure 8; Table II). In sharp contrast, although the mutant β 541 receptors did not induce the formation of dispersed colonies in agar, they were able to mediate clonal suppression of WEHI-3B D+ with a normal dose-responsiveness to hGM-CSF (Figure 9). These data suggest that WEHI-3B D+ cells can be clonally suppressed by pathways not operative in M1 cells and that sequences between the transmembrane domain and amino acid 541 of β c can mediate clonal suppression in some, but not other, types of leukaemic cells. The β 541 receptors were also able to mediate an increase in the expression of macrophage-specific markers (Table III) and changes in morphology similar to that seen with the β 626 mutant receptor. The β 461 receptor mutant in WEHI-3B D+ cells behaved essentially the same as in M1 cells, with no response to hGM-CSF by any of the criteria tested. The inconsistent effects of human GM-CSF on the WEHI-3B D+ cells at high concentrations may be mediated by interaction of hGMR α with endogenous mouse β c as they are also seen

Fig. 9. Differentiation of WEHI-3B D+ cells expressing hGMR α -chain and mutant β c in response to hGM-CSF. WEHI-3B-D+ cells expressing hGMR α -chain and either β 783, β 626, β 541 or β 461 were cultured as described previously with various concentrations of hGM-CSF. Cultures were scored for percentage of differentiated colonies at day 7. The left-hand panels show the percentage of colonies containing differentiated cells; the right-hand panels show the total number of colonies per plate. $\circ$, $\bullet$ indicate the responses of two independent clones; the bars to the right of each panel indicate the ability of the cells to respond to a maximal concentration of G-CSF.

in WEHI-3B D+ cells expressing only hGMR α (Figures 5 and 9).

Activation of JAK2 and STAT5b by β c truncation mutants in M1 cells

To assess the ability of the receptor mutants to activate JAK2 (via phosphorylation on tyrosine residues) and downstream signalling components such as the signal transducers and activators of transcription (STATs), Western blot analysis was performed on cell lysates (from stimulated and unstimulated M1 cells) that had been immunoprecipitated with an antibody to phosphorylated tyrosine residues. In M1 cells expressing hGMR α alone, stimulation with hGM-CSF did not increase the level of tyrosine-phosphorylated JAK2 or STAT5b (Figure 10, lanes 1 and 2). In M1 cells expressing hGMR α and either β c, β 783, β 626 or β 541, hGM-CSF induced an increase in the level of JAK2 phosphorylation compared with the unstimulated controls (Figure 10, lanes 3–10). However, M1 cells expressing hGMR α and β 487 or β 461 did not show an induction of JAK2 phosphorylation in response to hGM-CSF (Figure 10, lanes 11–14). STAT5b tyrosine phosphorylation was increased in response to hGM-CSF stimulation in M1 cells expressing wild-type β c and truncation mutants β 783 and β 626. Interestingly, although the truncation mutant β 541 was able to stimulate JAK2 phosphorylation, it was not able to stimulate an increase in the level of tyrosine phosphorylation of STAT5b (Figure 10, lanes 9 and 10). As would be expected for receptor mutants that could not activate JAK2, truncation mutants

Fig. 10. Phosphorylation of JAK2 and STAT5b in M1 cells expressing hGMR α and β c (wild-type and mutants) in response to saline (–) or hGM-CSF (+). Lysates from M1 cells expressing hGMR α -chain (lanes 1 and 2), hGMR α -chain and either β c (lanes 3 and 4), β 783 (lanes 5 and 6), β 626 (lanes 7 and 8), β 541 (lanes 9 and 10), β 487 (lanes 11 and 12) or β 461 (lanes 13 and 14) were prepared and immunoprecipitated with an anti-phosphotyrosine antibody as described in Materials and methods. Immune complexes were separated on an SDS–acrylamide gel and analysed by Western blot with antibodies specific for JAK2 and STAT5b. Immunoreactive bands corresponding to JAK2 (130 kDa) and STAT5b (95 kDa) were visualized with the ECL system.

β 487 and β 461 were unable to induce tyrosine phosphorylation of STAT5b in response to hGM-CSF.

Discussion

The present studies were aimed at defining the regions of the cytoplasmic domain of the human common β -chain (β c) of the GM-CSF receptor complex that are required to transmit a differentiating signal to myeloid cells. These regions could then be compared with those already defined in β c for proliferative and cell survival signalling and the induction of intracellular tyrosine phosphorylation. They could also be compared with regions of the G-CSF and erythropoietin receptor cytoplasmic domains that have been proposed to mediate differentiating signalling (Dong *et al.*, 1993; Fukunaga *et al.*, 1993; Maruyama *et al.*, 1994; Yoshikawa *et al.*, 1995).

Human β c receptors or truncation mutants were co-transfected with hGMR α into two different murine myeloid leukaemic cell lines, M1 and WEHI-3B D+, to map the regions of the receptor required for myeloid differentiation (Figures 1 and 11) and test several hypotheses. The M1 cells used for this study can be induced to macrophage differentiation by leukaemia inhibitory factor (LIF) or interleukin-6 (IL-6) with profound clonal suppression but murine GM-CSF has no such action and the cells do not display endogenous GM-CSF receptors. In contrast, WEHI-3B D+ cells do display endogenous GM-CSF receptors and are weakly induced to differentiate by murine GM-CSF. They are more strongly induced by G-CSF and IL-6 but are unaffected by LIF and do not display LIF receptors.

Can the human GM-CSF receptor function to signal differentiation and clonal suppression in a murine cell line, particularly one that does not constitutively express GM-CSF receptors?

M1 cells co-transfected with wild-type hGMR α and h β c were clearly responsive to hGM-CSF with the induction of morphological differentiation to macrophages, the capacity of such cells to migrate through agar, the induction of the macrophage surface markers Fc γ receptor, Mac-1, c-fms, F4/80 and the granulocyte marker Gr-1 as well as a profound clonal suppression of proliferative capacity.

These effects were all equivalent to the strong responses to LIF and IL-6 seen in these cells and could be achieved with relatively low concentrations of human GM-CSF.

Is the differentiation response determined by the type of receptor utilized or by intrinsic cellular programming?

The response of the transfected M1 cells to human GM-CSF was nearly identical to the responses to LIF and IL-6. However, when WEHI-3B D+ cells were transfected with human GM-CSF receptor α - and β -chains, the response to human GM-CSF was different from that mediated by endogenous G-CSF and GM-CSF receptors. The differentiation response was stronger with human GM-CSF as assessed by morphological differentiation and the induction of the cell surface markers Mac-1, F4/80 and Gr-1. More strikingly, human GM-CSF induced a profound clonal suppression of proliferative capacity in the transfected WEHI-3B D+ cells that was not observed with G-CSF at any concentration. In fact, the response of these cells to human GM-CSF was very similar to the response of transfected M1 cells with the exception that Fc γ receptors and c-fms were not elevated, possibly as a result of their already high levels in uninduced WEHI-3B D+ cells. The reason for the different response of transfected WEHI-3B D+ cells to murine GM-CSF or G-CSF compared with human GM-CSF is not yet clear. It may be that hGM-CSF receptors are expressed at a higher level than endogenous G-CSF or GM-CSF receptors and hence deliver a stronger differentiative stimulus or that introduced hGM-CSF receptors activate different cellular pathways to those activated by the endogenous receptors. In fact, the response of transfected WEHI-3B D+ cells to hGM-CSF was very similar to the responses to LIF of WEHI-3B D+ cells transfected with the LIF receptor (Tomida, 1995).

Does the introduction of human GM-CSF receptors lead to constitutive differentiation induction or dampen the response to other differentiation inducers?

Since some signal transduction molecules (such as JAKs) associate constitutively with receptor cytoplasmic domains it is possible that receptor overexpression *per se* could lead

Fig. 11. Critical regions of the cytoplasmic domain of human βc required for different biological functions in CTLL-2 cells or Ba/F3 (induction of cell cycle, cell survival and negative regulation of proliferation as well as activation of the ras pathway) and M1 and WEHI-3B D+ cells (clonal suppression, differentiation). The amino acid numbering system is from the first amino acid of the leader sequence with 461 being the first amino acid of the cytoplasmic tail. Dark boxes delineate the essential region between a truncation mutant that is active and one that is not. The hatched box indicates a region capable of clonal suppression in WEHI-3B D+ cells (but not M1). White boxes delineate regions that are not essential but which enhance the biological response [data from the present paper and Sakamaki *et al.* (1992), Sato *et al.* (1993) and Kinoshita *et al.* (1995)].

to differentiative signalling or, conversely, that depletion of signal transduction molecules from other endogenous receptors might diminish responses mediated by endogenous receptors. We found no consistent evidence for either event. The responses of transfected M1 cells to LIF or of transfected WEHI-3B D+ cells to G-CSF were essentially the same as for parental cells. While some clones of transfected cells did show higher levels of spontaneous differentiation, this was not a consistent observation and did not correlate with expressed receptor numbers, suggesting that other mechanisms were involved.

Are the receptor cytoplasmic domains required for different aspects of cellular differentiation or clonal suppression the same?

The induction of differentiation in haematopoietic cells has often been thought of as an all-or-none process leading ultimately to the clonal suppression of proliferative potential in mature cells such as granulocytes, macrophages and erythroid cells. In the present study we have examined several different aspects of cellular differentiation: the formation of dispersed colonies in agar as a measure of macrophage migratory activity; clonal suppression of proliferative capacity; the induction of macrophage

cell surface markers (differentiation antigens); morphological differentiation; and adherence to plastic. The results have shown that each of these processes is dependent on the co-expression of βc (with the GM-CSF receptor α -chain) but that the regions of the cytoplasmic domain of βc required are, to some extent, cell-line specific and different for the different aspects of cellular differentiation.

For M1 cells all the measured aspects of differentiation were qualitatively correlated and required, as a minimum, amino acid sequences between the transmembrane domain and amino acid 626 of βc . However, there were quantitative differences in that the $\beta 626$ mutant showed an unaltered dose-responsiveness for GM-CSF-induced clonal suppression but a dramatically decreased responsiveness to GM-CSF for the induction of macrophage migratory activity. Although titration curves were not performed, it was also clear that M1 cells expressing $\beta 626$ could also be fully induced by GM-CSF for the induction of macrophage differentiation markers, macrophage morphology and adherence to plastic. Shorter truncations of βc (e.g. $\beta 541$ or shorter) were unable to mediate any of the differentiating effects of GM-CSF in M1 cells.

In contrast, transfected WEHI-3B D+ cells expressing $\alpha\beta 626$ completely lost the capacity to be induced by GM-CSF for macrophage migratory activity in agar but retained an unaltered responsiveness to GM-CSF for the induction of clonal suppression and could be fully induced for differentiation markers. Indeed, even the shorter truncated form $\alpha\beta 541$ fully retained the capacity for GM-CSF-dependent induction of clonal suppression and differentiation markers in WEHI-3B D+ but not M1 cells.

These data suggest that the induction of cellular differentiation may not be a single process but rather the result of cumulative signals emanating from different regions of βc that impinge on critical cell signalling intermediates whose pool size or responsiveness is cell-specific. One model that could rationalize these observations is shown in Figure 12. At least two signals from βc cooperate to induce macrophage migratory activity (one emanating from $\beta 541$ –626 and one from $\beta 626$ –783) and these signals act on a critical signalling intermediate (shown in white in Figure 1) whose pool size is different in M1 and WEHI-3B D+ cells. The pool size is large enough in M1 cells so that maximum signalling from $\beta 541$ –626 is sufficient to induce macrophage migration, but in WEHI-3B D+ cells the pool size is small and requires signalling from both cooperative pathways. An exactly parallel situation exists for the induction of clonal suppression, surface markers and morphological differentiation except that the signalling pathways are different, emanate from different regions of the receptor (β TM-541 and $\beta 541$ –626) and act on different signalling intermediates with reversed pool sizes with respect to M1 and WEHI-3B D+ cells (Figure 12). In this case signals from β TM-541 are sufficient to activate the WEHI-3B D+ pool of signalling molecules while cooperative signals from $\beta 541$ –626 are required to activate the smaller pool in M1 cells. Depending on the abnormalities in particular leukaemic cell lines, this model could also explain reported instances of clonal suppression without evident effects on cellular differentiation (Begley *et al.* 1987; Maekawa and Metcalf, 1987).

Fig. 12. Multiple pathways are involved in the induction of macrophage differentiation by GM-CSF in M1 and WEHI-3B D+ cells. The lack of correlation between the formation of dispersed colonies in agar (macrophage migration) and clonal suppression/macrophage surface markers/morphology/adherence suggests that these might be signalled in different ways. The cell line differences suggest that critical signalling intermediates may have different pool sizes in M1 and WEHI-3B D+ cells for these different aspects of macrophage differentiation.

How do these results compare with previously defined functional regions in β c and other haematopoietic receptors?

Sakamaki *et al.* (1992) originally suggested that β c truncated after box-1 (533 in our numbering system) was still able to signal proliferation in Ba/F3 and CTLL-2 transfectants but that downstream regions including box-2 (to 559) were also important for a full proliferative signal. Subsequent experiments (Kinoshita *et al.*, 1995) showed that this proliferation was transient and that most cells had died by apoptosis within 60 h of culture. On the other hand, longer forms of β c (778 or longer) allowed extended hGM-CSF-stimulated proliferation, suggesting that a survival signal was transduced in this region. Our proliferation assays were scored at 48–72 h and for CTLL-2 cells we saw sustained proliferation with β c truncations as short as 626, suggesting that the long-term cell survival signal is mediated between positions 559 and 626 (Figure 11). In fact, β c truncations to 783 or 626 conferred on CTLL-2 cells a ~10-fold increased responsiveness to hGM-CSF-induced cell survival/proliferation compared with full-length β c, suggesting that the region of β c between 783 and the C-terminus mediates a negative regulatory region. Such a negative modulating region has also been defined for the human erythropoietin receptor (Klingmuller *et al.*, 1995; Yi *et al.*, 1995) and the IL-3 receptor β c chain (Yi

et al., 1993) as the result of binding Hemopoietic Cell Phosphatase (HCP or SH-PTP1).

GM-CSF-induced phosphorylation of JAK2 has been implicated in the mitogenic pathway in BA/F3 cells (Watanabe *et al.*, 1996) and we found that JAK2 was tyrosine-phosphorylated in response to GM-CSF for all β c mutants that induced proliferation in transfected CTLL-2 cells (Figure 10). However, since the β 541 mutant was able to mediate JAK2 phosphorylation but not differentiation in M1 cells, JAK2 phosphorylation appears to be insufficient for differentiation induction. Phosphorylation of STAT5 is required for its ability to bind DNA and induce transcription (Gouilleux *et al.*, 1994) and STAT5 may have a role in IL-3-induced cell proliferation (Mui *et al.*, 1996). In transfecting M1 cells we showed that all β c mutants that mediated GM-CSF-induced differentiation (β 626 or longer) were also able to mediate tyrosine phosphorylation of STAT5b (Figure 10). This agrees with recent results from Mui *et al.* (1995) who defined a region between amino acids 533 and 560 (in our numbering system) that was required for STAT5 phosphorylation. STAT3 phosphorylation has also been associated with gp-130-mediated differentiation in M1 cells (Yamanaka *et al.*, 1996), but a definitive role for STAT3/STAT5 phosphorylation in M1 cell differentiation will require further study.

Sakamaki *et al.* (1992) and Sato *et al.* (1993) showed that the major tyrosine phosphorylation, PI3 kinase activation, *shc*, *ras*, MAP kinase and *fos/jun* activation induced by human GM-CSF all required sequences from 642 to 778 while *pim-1* and *myc* induction required sequences only up to 533. However, more recent studies have shown that Tyr593 (in our numbering system) is important for GM-CSF-mediated binding of *shc* to β c (Pratt *et al.*, 1996), indicating that regions upstream of 642 may also be able to activate this pathway.

We found that sequences truncated to 541 could induce clonal suppression and differentiation markers in WEHI-3B D+ cells and that sequences truncated to 626 could induce differentiation and clonal suppression in M1 cells and WEHI-3B D+ cells. These results separate at least some aspects of differentiation induction in these cell lines from the region of β c identified as being important for activation of the *shc-ras-raf*-MAP kinase pathway. However, as we have noted, sequences of β c downstream of 626 do enhance the dose-responsiveness of differentiation and are specially important for full differentiation induction in WEHI-3B D+ cells. β c truncated to 783 was as effective as wild-type β c in differentiation induction, showing that the terminal 114 amino acids are not required. The regions C-terminal to box-1 and box-2 of the G-CSF and erythropoietin receptors have also been shown to be required for induced granulocyte and erythroid cell differentiation (Dong *et al.*, 1993; Fukunaga *et al.*, 1993; Maruyama *et al.*, 1994; Yoshikawa *et al.*, 1995) and specific differentiated gene function (Ziegler *et al.*, 1993; Baumann *et al.*, 1994).

These data support a model of GM-CSF-dependent signal transduction where multiple different signalling pathways are initiated from distinct regions of β c (Figure 11). Regions to 541 are sufficient to signal clonal suppression and the induction of differentiation markers in WEHI-3B D+ but not M1 cells. Regions between 472

and 583 are sufficient to initiate short-term proliferation in Ba/F3 and CTLL-2 cells (Sakamaki *et al.*, 1992; Sato *et al.*, 1993) while regions between 559 and 626 appear to be required for cell survival and sustained proliferation (Kinoshita *et al.*, 1995 and present results). A negative regulatory element for the proliferative response, presumably involving tyrosine phosphatases, occurs between amino acids 783 and 897. Regions to 626 are required to induce macrophage migratory activity in cell lines but their effects are enhanced by regions between 626 and 783. Regions between 642 and 778 are required for induction of the *shc-ras-raf*-MAP kinase and PI-3 kinase pathways (Sato *et al.*, 1993). Further delineation of these distinct regions of the cytoplasmic domain of βc and the biochemical pathways they activate should, in the future, allow precise targeting of the varied cellular responses to cytokines.

Materials and methods

Cell lines and growth factors

M1 (Metcalf *et al.*, 1988) and WEHI-3B D+ cells (Metcalf, 1979) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). CTLL-2 cells (Cerottini *et al.*, 1974) were grown in DMEM supplemented with 10% (v/v) FCS, 10 mM HEPES, 22 mM glucose, 2 mM glutamine, 0.5 mM arginine, 0.3 mM asparagine, 120 μ M folic acid, 50 μ M 2-mercaptoethanol and mIL-2. Cells were maintained in a humidified atmosphere containing 10% CO₂ and passaged twice weekly.

Recombinant human GM-CSF and human G-CSF were obtained from Amgen (Thousand Oaks, CA, USA), murine IL-2 from R&D Systems (MN, USA) and murine LIF was produced using the pGEX system as described (Gearing *et al.*, 1989b).

Expression constructs

Truncated hGMR β -chains were generated using the polymerase chain reaction (PCR). Briefly, antisense oligonucleotides which contained a stop codon to replace amino acids 783 (β 783), 626 (β 626), 541 (β 541), 487 (β 487) and 461 (β 461) followed by an *Xho*I cleavage site and sense oligonucleotides which contained a restriction enzyme site (*Bgl*II or *Bss*HII) upstream of the truncation were synthesized using an Applied Biosystems PCR-MATE 391 DNA Synthesizer (Applied Biosystems, Foster City, CA, USA). PCR was carried out in a final reaction volume of 100 μ l under the following reaction conditions: 20 mM Tris-HCl, pH 8, 10 mM KCl, 6 mM (NH₄)₂SO₄, 0.1% (v/v) Triton X-100, 2 mM MgCl₂, 0.2 mM each dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 4 ng of each oligonucleotide, 5 U *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and 100 ng template DNA. DNA was amplified using conditions of 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, 60°C annealing for 1 min and 72°C extension for 90 s on a Perkin Elmer Cetus DNA Thermal Cycler 480 (Perkin Elmer Cetus, Foster City, CA, USA). The plasmid containing the hGMR β -chain cDNA (a gift from Dr P.Lock) and PCR products were digested with *Xho*I and either *Bss*HII (β 461, 487 and 541) or *Bgl*II (β 626 and 783), separated on a 1% (w/v) agarose gel and purified using the GeneClean II Kit (Bio 101 Inc., Vista, CA, USA) according to the manufacturer's instructions. Receptor cDNA fragments were cloned back into the β -chain cDNA and sequenced using standard protocols to ensure that no errors had been introduced during the PCR. Wild-type and mutant β -chains were then subcloned into the *Bst*XI site of the pEF-BOS plasmid (Mizushima and Nagata, 1990) using *Bst*XI adaptors (Invitrogen, San Diego, CA, USA).

Plasmids encoding resistance genes for selectable markers puromycin (a gift from Professor S.Cory), neomycin and hygromycin (gifts from Dr P.Lock) transcribed from the PGK promoter were linearized with *Sca*I and receptor cDNAs in pEF-BOS were linearized with *Hind*III before transfection.

Cell transfection

Stable cell lines expressing the human GMR α -chain alone, α -chain plus wild-type β -chain or α -chain plus each of the truncated β -chains were transfected by electroporation. Cells were washed once in phos-

phate-buffered saline (PBS) and resuspended at 5×10^6 cells/ml. An aliquot of the cells (4×10^6) was transferred into 0.4 mm electroporation cuvettes containing 20 μ g of expression plasmid and 2 μ g of plasmid encoding genes for selectable markers. DNA and cells were incubated at room temperature for 10 min before electroporating at 270 V and 960 μ F in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). Cells were then incubated for a further 5 min at room temperature before adding 1 ml of medium and layering over 2 ml FCS. Cells were centrifuged through the FCS to remove cell debris, resuspended in 100 ml culture medium and dispensed into four 24-well tissue culture plates. The cells were returned to the incubator for 48 h before selection by the addition of puromycin (Sigma Chemicals, St Louis, MO, USA) to a concentration of 20 μ g/ml for both M1 and WEHI-3B D+ cells and 5 μ g/ml for CTLL-2 cells (single transfectants). Double-transfectants were selected with 20 μ g/ml puromycin and either 500 μ g/ml hygromycin-B (Boehringer Mannheim Australia, Castle Hill, NSW, Australia) for M1 cells or 1.5 mg/ml geneticin (Gibco-BRL, Gaithersburg, MD, USA) for WEHI-3B D+ cells and 5 μ g/ml puromycin with 200 μ g/ml hygromycin for CTLL-2 cells. Clones of proliferating cells were visible after 10–14 days of selection and were expanded to test for receptor expression. Individual clones were obtained by limiting dilution or by picking of individual colonies from agar cultures.

Binding studies

Escherichia coli-derived human GM-CSF in pure form was a kind gift from Schering Corporation, NJ. 10 μ g of purified protein in 0.2 M sodium phosphate buffer, pH 7.4, were radio-iodinated using a modified iodine monochloride technique (Nicola and Metcalf, 1988) and separated from unreacted iodide by gel filtration on Sephadex G-25M (Pharmacia, Uppsala). This material had a specific radioactivity of 4.5×10^5 c.p.m./pmol as determined by self-displacement analysis (Nicola and Metcalf, 1988).

Transfected cells ($1\text{--}4 \times 10^6$ /point) were incubated in duplicate with increasing concentrations of [¹²⁵I]hGM-CSF ($2 \times 10^3\text{--}2 \times 10^6$ c.p.m.) with or without unlabelled hGM-CSF (5 μ g/ml final concentration) in HEPES (20 mM) buffered, pH 7.4, RPMI medium containing 10% FCS (100 μ l) at 4°C for at least 3 h. The reaction mixture was then layered over FCS (200 μ l) in plastic microcentrifuge tubes (Elkay 0021, Melbourne) and centrifuged at 13 000 g for 10 s. The cell pellet and supernatant were recovered separately by cutting the tube with a scalpel blade and each counted in a Packard gamma-counter. Specific binding was determined as the difference in counts between parallel sets of tubes with or without unlabelled hGM-CSF and the data were analysed by the LIGAND program (Munson and Rodbard, 1980) for both 1-site and 2-site binding interactions. The program generates a statistical evaluation of the goodness of fit for 1-site versus 2-site models and the 2-site analysis was only employed if $P < 0.05$. The data are presented as Scatchard plots (specific bound/free GM-CSF versus bound GM-CSF) although curve fitting was performed on untransformed data.

Biological assays

The proliferation of CTLL-2 cells in response to cytokines was measured in a Lux HL-A plate (Nunc Inc., Roskilde, Denmark). Cells were washed in DMEM containing 50 μ M 2-mercaptoethanol, 2 mM glutamine and 20% (v/v) FCS to remove IL-2 and cultured using 200 cells/well in 15 μ l medium containing serial 2-fold dilutions of 5 μ l either murine IL-2 or human GM-CSF. After 48 h incubation at 37°C in a fully humidified incubator containing 10% CO₂ in air, the number of viable cells remaining was assessed microscopically.

To assess the differential response of M1 and WEHI-3B D+ cells to cytokine stimulation, 100 cells were cultured in 35 mm Petri dishes containing 1 ml DMEM supplemented with 20% (v/v) FCS, 0.3% (w/v) agar and 0.1 ml serial dilutions of LIF, GM-CSF or G-CSF. After 7 days' incubation at 37°C in a fully humidified atmosphere containing 10% CO₂ in air, colonies of M1 and WEHI-3B D+ cells were counted and scored for differentiation. Colonies were scored as differentiated if they were composed entirely of dispersed cells or exhibited a corona of dispersed cells surrounding a more tightly packed central core.

Differentiation of both M1 and WEHI-3B D+ cells was also assessed in liquid culture. Cells (1×10^4 /ml) were cultured in DMEM supplemented with 10% (v/v) FCS and stimulated with either saline, 10^3 U/ml mLIF (M1 cells), hG-CSF (WEHI-3B D+ cells) or 10^3 U/ml hGM-CSF for 4 days at 37°C. Adherent and non-adherent cells were harvested and centrifuged onto glass slides in a Cytospin centrifuge (Shandon Elliott). Slides were then stained with May-Grünwald Giemsa and assessed for differentiation on the basis of a morphological change from blast cell towards a macrophage phenotype.

FACS analysis

To assess further the differentiation of M1 and WEHI-3B D+ cells in response to cytokines, expression of macrophage and granulocyte cell surface markers Mac-1 (Springer *et al.*, 1979), FcγR1 (Unkeless, 1979), c-fms or macrophage colony stimulating factor receptor (Anderson *et al.*, 1982), Gr-1 (Hestdal *et al.*, 1991) and F4/80 (Austyn and Gordon, 1981) were examined using flow cytometry. Adherent and non-adherent cells were harvested by treatment with 40 mM EDTA at room temperature for 5 min. Cells were washed once in staining solution [PBS containing 1% (v/v) FCS]. Cells (1×10^6) were incubated at 4°C for 30 min with 50 μl rat anti-Fc receptor-blocking antibody to block non-specific Fc binding of the test antibodies, 1 μg of biotinylated anti-Mac-1 (PharMingen, San Diego, CA, USA), Gr-1 or F4/80 (a gift from Dr Andreas Strasser) and 0.2 μg anti-c-fms (Oncogene Science Inc., Manhasset, NY, USA) or 1 μg corresponding biotinylated isotype control antibody (PharMingen). Cells stained for FcγR expression were resuspended in staining solution with 1 μg biotinylated anti-FcγR antibody (PharMingen) with no blocking antibody or 1 μg corresponding biotinylated isotype control antibody (PharMingen). After washing twice with 2 ml staining solution, cells were incubated at 4°C for 30 min in 100 μl of staining solution containing either 2 μg fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin (for c-fms; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or streptavidin-phycoerythrin (Caltag Laboratories, San Francisco, CA, USA) diluted 200-fold (for Mac-1, FcγR, F4/80 and Gr-1). Cells were washed twice with staining solution and resuspended in 500 μl staining solution containing 1 μg/ml propidium iodide to assess viability. FACS analysis was performed with a Becton Dickinson FACScan using FACScan software (Becton Dickinson, San Jose, CA, USA).

Immunoprecipitation and Western blot analysis

Washed cells were resuspended at 1×10^7 cells/ml in DMEM with 10% (v/v) FCS and incubated at 37°C for 60 min. After stimulation with 10^4 U/ml hGM-CSF or saline for 10 min, the reaction was stopped by addition of sodium vanadate (Sigma Chemicals, St Louis, MO, USA) to a final concentration of 1 mM and placing the cells on ice. Cells were washed once with 5 ml of ice-cold normal saline containing 1 mM sodium vanadate and lysed in 250 μl cold lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor cocktail (Boehringer Mannheim, Castle Hill, NSW, Australia; 1 tablet in 50 ml extraction buffer) for 5 min on ice. Cell lysates were centrifuged at maximum speed in an Eppendorf centrifuge for 5 min at 4°C to remove insoluble material. The supernatants were then incubated with 5 μg of anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., NY, USA) and protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) overnight at 4°C. Sepharose beads were pelleted by centrifugation and washed twice with 1 ml cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor cocktail as above). Pelleted beads were resuspended in an equal volume of reducing SDS sample buffer (containing 2-mercaptoethanol) and boiled for 5 min. Samples were then centrifuged at maximum speed for 5 min and the supernatants electrophoresed on 4–15% Tris-glycine Ready-gels (Bio-Rad Laboratories, Hercules, CA, USA).

Following transfer to polyvinylidene difluoride-plus membrane (Micron Separations Inc., Westborough, MA, USA), the membrane was incubated in blocking buffer [normal saline containing 1% (w/v) bovine serum albumin and 0.1% (v/v) Tween-20] for 1 h at room temperature. Membranes were then incubated with rabbit polyclonal antisera to JAK2 and STAT5b (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) diluted to 1 μg/ml in blocking buffer for 2 h at room temperature. After washing three times with blocking buffer, membranes were incubated with goat anti-rabbit antisera conjugated to horseradish peroxidase (Transduction Labs, Lexington, KY, USA), and diluted in blocking buffer according to the manufacturer's instructions, for 1 h at room temperature. Following several washes in normal saline containing 0.1% Tween-20, membranes were developed with the ECL system (Amersham Life Science, UK) and exposed to Hyperfilm (Amersham) according to the manufacturer's instructions.

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