TIMELINE

The colony-stimulating factors and cancer

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Abstract | The four colony-stimulating factors (CSFs) are glycoproteins that regulate the generation and some functions of infection-protective granulocytes and macrophages. Recombinant granulocyte-CSF (G-CSF) and granulocyte–macrophage-CSF (GM-CSF) have now been used to increase dangerously low white blood cell levels in many millions of cancer patients following chemotherapy. These CSFs also release haematopoietic stem cells to the peripheral blood, and these cells have now largely replaced bone marrow as more effective populations for transplantation to cancer patients who have treatment-induced bone marrow damage.

When I began my career in leukaemia research in 1954, most workers in the field were searching for human leukaemia viruses. My interest in the quite different field of blood cell regulators had been aroused by work with tumours of endocrine target tissues, such as the thyroid or breast[1]. In elegant studies, Furth had shown that if mice were subjected to a sustained imbalance in hormones that favours cell proliferation, tumour development occurred in a stepwise fashion in the target tissues[2,3]. When thinking about how leukaemia might initiate, I was intrigued by the ideas of Furth in his 1954 essay: "On the basis of events with other regulated cells it can be postulated that a permanent disturbance of the homeostatic balance might result in leukaemias in which the proliferating cells are essentially unaltered, and which could be controlled at their inception by restoration of the deranged equilibrium of the regulatory forces" (REF. 3). In the context of leukaemia, although common sense said that regulators must exist to control white blood cells, unfortunately nothing was known about the possible nature of these regulators.

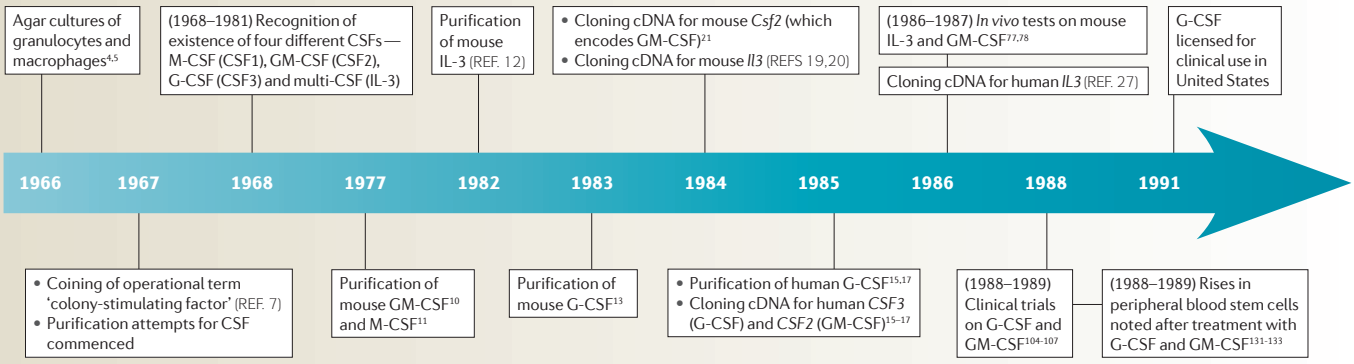
The colony-stimulating factors

Before the 1960s, many investigators had performed experiments in intact animals to discover possible regulators of white blood cell homeostasis, but nothing of substance had been observed. The situation changed dramatically in 1965–1966 when two groups simultaneously developed methods for growing colonies of white blood cells from mouse bone marrow or spleen cells in semi-solid agar and, later, in methylcellulose cultures[4,5] (TIMELINE). The colonies, as initially grown, contained maturing neutrophilic granulocytes (hereafter simply called granulocytes or neutrophils) and/or macrophages. The remarkable features of these colonies were that they were clones derived from single precursor cells (later termed progenitor cells (BOX 1)) and that the formation, number and size of colonies were absolutely dependent on the amount of cells, tissue extracts or medium conditioned by various tissues that were added to the cultures[4,6]. The culture system was dependent on the presence of an unknown active factor(s) (which was given the operational term, colony-stimulating factor; CSF)[7] that was needed to stimulate cell division. Subsequent efforts succeeded in growing similar colonies from human bone marrow cells using underlayers of white blood cells as 'feeder layers' that provided a source of the as yet unknown CSF[8].

Initial studies indicated that CSF was probably not a virus that had transformed bone marrow cells (at that time, only transformed cells were believed capable of proliferation in agar medium), was not a trivial nutritional material and was probably a protein. Efforts to purify CSF occupied many laboratories during 1968–1985. Initially human urine was used as a source material[9], then mouse organ- or cell line-conditioned medium and, eventually, similar media from human cells or human tumour cell-conditioned media were used. The task proved to be formidable. It slowly became inescapable that there was not a single CSF but, in fact, there were four quite different glycosylated CSF proteins each with differing colony-stimulating activity. Separating and purifying these four CSFs were rendered more difficult by variable glycosylation of the CSFs and the minute amounts of CSF in tissues. The four CSFs were given working names that indicated the most numerous type of colony stimulated — GM-CSF (also known as CSF2) stimulated granulocyte and macrophage colony formation; M-CSF (also termed CSF1) stimulated macrophage colony formation; G-CSF (also known as CSF3) stimulated granulocyte colony formation; and multi-CSF (now more commonly termed interleukin 3, IL-3) stimulated a broad range of haematopoietic cell colony types. In two instances, purification of more than 500,000-fold was required to derive pure CSF. The introduction and application of high-performance liquid chromatography was required for the eventual success of these purification attempts. Purification of mouse GM-CSF[10] and M-CSF[11] was reported in 1977, IL-3 in 1982 (REF. 12) and G-CSF in 1983 (REF. 13). Purification of human CSFs corresponding to the four mouse factors followed the purifications of mouse CSFs, and investigators made better use of human tumour cell lines as superior sources of CSFs[14–18].

Cloning of the cDNAs for all four CSFs, both mouse and human, from libraries using sequence-based probes or expression screening, occurred between 1984–1986 and were some of the earliest successes of molecular biology[16,19–27]. This was followed by the then difficult task of expressing the active protein in bacterial, yeast or mammalian cell systems but, eventually, adequate expression systems were developed.

Timeline | **Major events in the development of the colony-stimulating factors**



CSF, colony-stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte–macrophage-CSF; IL-3, interleukin 3; M-CSF, macrophage-CSF.

Biology of the CSFs

The CSFs are 18–70 kDa glycoproteins and, unlike the comparable erythroid regulator erythropoietin (EPO), the CSFs are active *in vivo* in both their glycosylated and non-glycosylated forms. The half-lives of glycosylated CSFs are longer than non-glycosylated CSFs, but are still only a matter of 1–6 hrs[28]. Unexpectedly, in studies between 1966 and 1984, the CSFs were found to be the products or potential products of most tissues and cell types in the body[28]. Normal levels of production were very low, even in the most active tissues, but CSF production was markedly inducible by microorganisms, endotoxin or foreign cells, which could increase production up to 1,000-fold in hours[28]. The CSFs can therefore be viewed as highly labile agents that are produced rapidly and to high levels in the presence of an inducing agent. M-CSF differs as it is produced in higher concentrations in a much more stable manner. The lability and the short lifespan of CSF molecules allow them to function as a highly responsive control system that regulates haematopoietic cells.

CSFs are secreted and enter the circulation in their active forms. The CSFs, in some contexts, resemble hormones, except that multiple cell types can produce CSFs. However, in other contexts, the CSFs are produced and act in a paracrine fashion in local microenvironments. Specific membrane receptors exist that are unique for each CSF and are displayed in small numbers on all maturation stages of cells in the granulocyte and monocyte-macrophage lineages, from committed progenitor cells to post-mitotic mature cells in the peripheral blood and tissues (BOX 1). CSFs are removed from the circulation by binding to specific membrane receptors displayed on granulocytic and macrophage cells. Then, after internalization of the CSF–receptor complex, CSFs are degraded[28,29]. Degradation and/or clearance of CSFs also occur in the liver and kidney[28].

CSFs proved to be notable because of their multiple actions on haematopoietic cells (BOX 2). CSFs are mandatory to stimulate the division of every appropriate lineage-committed haematopoietic progenitor cell and its progeny. Haematopoietic progenitors show great heterogeneity in their responsiveness to CSF stimulation, resulting in the characteristic sigmoid dose response curves shown in FIG. 1a as more progenitor cells are stimulated to commence proliferation[11–13,28] (BOX 1). Individual progenitor cells also vary in their proliferative activity but, in general, as CSF concentrations are increased, cell cycle times are shortened and there is a progressive increase in the number of progeny cells in each colony. The cells in developing granulocyte–macrophage colonies show progressive maturation with time, so higher CSF concentrations achieve higher numbers of mature progeny cells[28].

When bone marrow cultures were more carefully analysed and purified native or recombinant CSFs became available for more general use, it was recognized between 1977 and 1987 that agar cultures of bone marrow could sustain the development not only of granulocyte and macrophage colonies, but also colonies of eosinophils, megakaryocytes, mast cells, erythroid cells, blast cells and T and B lymphocytes. It also became apparent that the prefixes used to describe the CSFs under-represented their action. GM-CSF also stimulated eosinophil colony formation and, at high concentrations, megakaryocyte colony formation[30]. G-CSF had a minor capacity to stimulate some granulocyte–macrophage colonies[31]. M-CSF could stimulate granulocyte colony formation by some progenitor cells[28], and IL-3 could stimulate colony formation by progenitors of blast cells, granulocytes, macrophages, megakaryocytes, eosinophils, mast cells and erythroid cells[32]. When used in combination with an agent like stem cell factor (SCF, also known as KIT ligand), the CSFs could co-stimulate the proliferation of the earliest haematopoietic cells (BOX 1)[33–35].

It became apparent in 1967 that the CSFs were necessary for the survival of the progenitor (colony-forming) cells and their progeny in culture[36–38], and in 1990 withdrawal of CSF was shown to lead to death from apoptosis[39]. This initially prompted some to postulate that the CSFs had only survival effects and that cells could then proliferate spontaneously. This improbable suggestion was discounted by the persistence of CSF dependency for the proliferation of cell lines in which survival had been ensured by overexpression of BCL2 (REF. 40) and by the characterization of some of the intracellular mitotic signals (including JAK–signal transducer and activator of transcription (STAT) and cyclin activation) that are initiated when CSFs interact with their membrane receptors[41].

More controversial have been the findings that CSFs seem to be able to initiate maturation events in leukaemic cell lines[13,42] and possibly, at times, to dictate commitment decisions in granulocyte–macrophage precursors *in vitro*[43,44]. These CSF actions require further elucidation because some maturation

was observed in a BCL2-immortalized cell line in the absence of CSFs[45], and CSFs can stimulate cell proliferation in unusual cell types following the insertion and expression of CSF receptors into such cells[46]. This type of stimulation, however, does not alter the phenotype of the cells that respond to CSFs.

Finally, CSFs clearly have the capacity to stimulate the functional activity of mature cells. For example, GM-CSF can stimulate mature neutrophils to exhibit chemotaxis, enhance oxidative metabolism, enhance antibody-dependent phagocytosis and killing of microorganisms, and produce various regulatory proteins. Similar actions have been documented for eosinophils and monocytes and these actions have been noted both *in vitro* and *in vivo*. A similar range of actions has been documented for G-CSF, M-CSF and IL-3 acting on mature neutrophils or monocyte-macrophages[28,47–51].

It was initially puzzling how one agent that acted at very low molar concentrations on a few hundred receptors present on responding cells could induce such diverse changes[52]. This problem was made more complex by the recognition that only a single type of receptor existed for each CSF and that receptors for all four CSFs can coexist on most granulocytic and macrophage cells. The problem was resolved in the early 1990s when the specific membrane receptors for each CSF were characterized and cloned. The membrane receptors in their simplest forms such as those for G-CSF are homodimers[53], but the receptor chains can be arranged in more complex forms for agents such as GM-CSF for which the heterodimeric receptor is arranged as a dodecameric complex[54]. All CSF receptors have specific regions in the cytoplasmic domain of their signalling chains that can initiate the different signalling events that are required to induce a varied range of biological responses[55–59].

In the period following the discovery of the CSFs, other regulators of haematopoietic populations were discovered, resulting in a confusing picture of the further potential redundancies or interactions in the control system. For example, granulocyte colony formation *in vitro* can be stimulated by G-CSF, GM-CSF, M-CSF, IL-3, SCF, IL-6 and weakly by IL-11 (REF. 28). In particular, the CSFs seemed to have many biological actions that were potentially overlapping or redundant, and it required gene knockout studies in mice in the mid-1990s to establish that each CSF did, in fact, have actions that were exclusive to that CSF.

For example, G-CSF was clearly responsible for formation of 75% of the granulocytes under basal conditions[60]. GM-CSF, by contrast, did not seem to influence mature cell numbers. Instead, it was essential for the functional activity of macrophages, particularly those in the lung. In mice, the absence of GM-CSF or its receptor leads to alveolar proteinosis — a lung disease caused by the failure of local macrophages to eliminate surfactant[61,62], and the same disease state has been noted in humans who produce neutralizing autoantibodies against GM-CSF[63]. M-CSF was necessary for the formation and function of the major macrophage populations and, strangely, was necessary also for tooth eruption and successful pregnancy[64–66]. IL-3 was expected to be an important regulator but mice lacking IL-3 receptors showed no obvious changes in haematopoiesis[67,68]. Later studies in mice showed that IL-3 had substantial actions in producing satisfactory mast cell and basophil responses to parasites[69] and hapten-specific delayed-type hypersensitivity responses[70].

Knockout studies on the CSFs and other regulators have shown that each regulator has some unique actions *in vivo* but, importantly, the design of the body often requires synergistic actions between two or more regulators on many haematopoietic cells. For example, the dramatic effects of G-CSF *in vivo* (see below) require the synergistic action of SCF[71], and strong synergy is observable on granulocyte–macrophage progenitor cells *in vitro* between GM-CSF

Box 1 | **The cellular basis of blood cell formation**

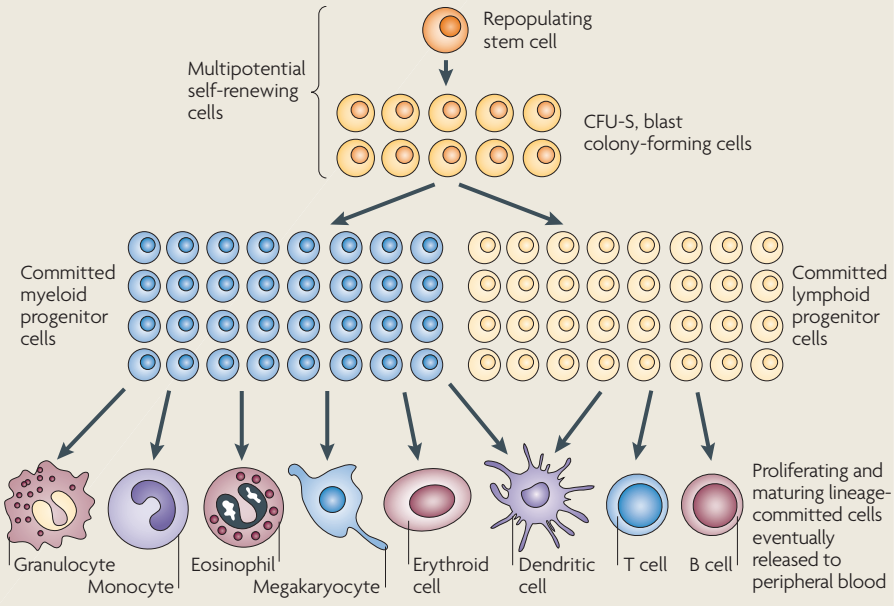
The road map of haematopoiesis

Stratified hierarchy of haematopoiesis. Three sequential classes of increasingly numerous ancestors exist in the bone marrow that generate maturing blood cells (shown in the figure below)[28]. A major separation occurs in cells that are committed to the formation of myeloid cells and those committed to the formation of T and B lymphocytes. Dendritic cells can be derived from both groups[156]. Cells committed to one or the other group can have their lineage commitment switched artificially by overexpression of genes such as *GATA1* or *SPI1* (also known as *PU.1*)[157].

Responsiveness to regulators. Committed myeloid progenitor cells and their progeny can respond to a single colony-stimulating factor (CSF) regulator but proliferation is enhanced synergistically by combining regulators. Less mature precursors require co-stimulation by multiple regulators[28].

Common ancestors. Many granulocyte and macrophage precursors have common ancestral cells, as do many erythroid and megakaryocyte precursors.

Heterogeneity of individual cells. In each maturation category of granulocytes and macrophages there is much heterogeneity between individual cells in quantitative responsiveness to CSF stimulation and some cells respond better or only to one particular CSF[28].



and M-CSF or G-CSF, and on less mature blast colony-forming cells between G-CSF and SCF or SCF and IL-6 (REFS 34,72). Conversely, some combinations are inhibitory. For example, G-CSF inhibits megakaryocyte colony formation stimulated by SCF and EPO[73].

One picture that has emerged from the culture of mouse bone marrow cells is that lineage-committed progenitor cells can respond to single regulators but that more immature cells require two or more regulators acting in concert before proliferation occurs (BOX 1). There are exceptions and, for example, optimal proliferation of mature megakaryocyte progenitor cells requires SCF, IL-3 and EPO[74], as does the proliferation of subsets of apparently lineage-committed progenitor cells that co-fractionate after fluorescence-activated cell sorting (FACS) separation with stem cell and colony-forming unit, spleen (CFU-S, which are early progeny of stem cells) populations[75].

In vivo actions of the CSFs in mice

By the early 1980s, the growing evidence for the existence of multiple regulators of haematopoietic tissues raised the spectre that complex interactions between these regulators might dampen or prevent any one agent from eliciting measurable responses *in vivo*.

However, this was not the case when recombinant mouse CSFs became available for testing in the mid-1980s. Injection of CSFs in mice elicited responses that were qualitatively similar to the actions observed *in vitro*. Subcutaneous G-CSF injections administered twice daily elicited substantial rises in blood neutrophil levels within 4 days following increased production of granulocytes in the bone marrow[76]. Intraperitoneal injections of GM-CSF in mice had less effect on circulating white cell levels but strongly increased peritoneal macrophage numbers and proliferative activity[77]. Subcutaneous injections of mouse IL-3 increased bone marrow cellularity and particularly increased the numbers of mast cells in various tissues[78,79]. It was also evident that GM-CSF and IL-3 injections in mice increased the phagocytic activity of mature macrophages towards antibody-coated erythrocytes[77,78].

An obvious question to pose was: could CSF injections enhance resistance in mice to serious fungal or bacterial infections of the types that are encountered in patients with cancer following chemotherapy? This question was examined in mice at the time of the earliest clinical trials on CSFs. In particular, G-CSF injections were tested in multiple infectious disease models and were found to clearly enhance resistance to and survival from various infectious organisms[80–84]. An important conclusion from these studies was that CSF administration before challenge with infectious agents was highly effective, whereas if CSF was administered after infections were initiated, the protective effects were minimal and were only significant if combined with antibiotics.

Do excessive levels of CSF induce toxic effects in mice? Excess GM-CSF levels in transgenic mice or in mice repopulated by bone marrow cells that were engineered to overexpress GM-CSF caused excess numbers of granulocytes and macrophages to develop and induced a range of fatal inflammatory lesions in the lung, muscles, bowel and peritoneal cavity[85,86]. Similarly, in mice repopulated by marrow cells that expressed excess IL-3 levels, hyperproliferation of haematopoietic and mast cell populations occurred. This was associated with uncontrollable itching and scratching, probably because of mast cell degranulation in the skin[87]. Although repopulation of mice with bone marrow cells that expressed excess levels of G-CSF induced excessive granulopoiesis and very high granulocyte levels, these effects caused no apparent tissue damage[88]. In subsequent studies, this outcome was radically altered by knocking out the suppressor of cytokine signalling 3 (*Socs3*) gene. SOCS3 is one of the Socs family of cytoplasmic suppressors of cytokine-initiated receptor signalling and suppresses signalling from activated G-CSF receptors[89]. Mice lacking *Socs3* are hyper-responsive to G-CSF, and administration of normal doses of G-CSF caused hind limb paralysis and death in days owing to a massive accumulation of neutrophils in the spinal cord, liver, lungs and marrow[90]. The lack of toxicity of G-CSF in mice and presumably in humans is therefore dependent on the modulating effects of SOCS3.

CSFs and myeloid leukaemia

From studies in the early 1970s on the clonal culture of primary human myeloid leukaemia cells, it was established that chronic myeloid leukaemia (CML) cells formed large, apparently normal, granulocyte or granulocyte–macrophage colonies *in vitro*[91]. By contrast, acute myeloid leukaemia (AML) cells often failed to proliferate or, at best, formed small clusters of progeny *in vitro*[92]. The striking observation was that all CML and most AML cells remained wholly dependent on stimulation by CSF-containing material for proliferation *in vitro*, and this did not require large concentrations of CSF. This situation did not change when purified CSFs later became available[93]. This suggested that CSFs, at a minimum, might be co-factors in the development of myeloid leukaemia, if for no other reason than that they could supply the proliferative and survival stimuli for the clonal expansion of emerging leukaemia cells *in vivo*.

Do sustained excess levels of CSF lead to myeloid leukaemia development? This question has only been posed for GM-CSF. Although lifelong excess GM-CSF levels in transgenic mice were not leukaemogenic[94],

Glossary

Aplastic
Severely reduced cellular content

Commitment
The change, usually irreversible, when a multipotent cell generates or becomes a cell that expresses membrane markers and a gene programme restricting the cell to a particular lineage

Conditioned medium
Medium harvested after incubation of cultured cells or tissues

Febrile neutropenia
Condition of abnormally low blood neutrophil levels plus fever

Immortalization
A change rendering cells capable of proliferation for prolonged (perhaps unlimited) time periods, usually the cells are not neoplastic

Lineage
A subfamily of one type of haematopoietic cell

Maturation
The sequence of morphological and biochemical changes during which immature cells generate or become mature cells

Socs family
A family of cytokine (regulator)-induced cytoplasmic inhibitors of signalling from regulator-activated membrane receptors

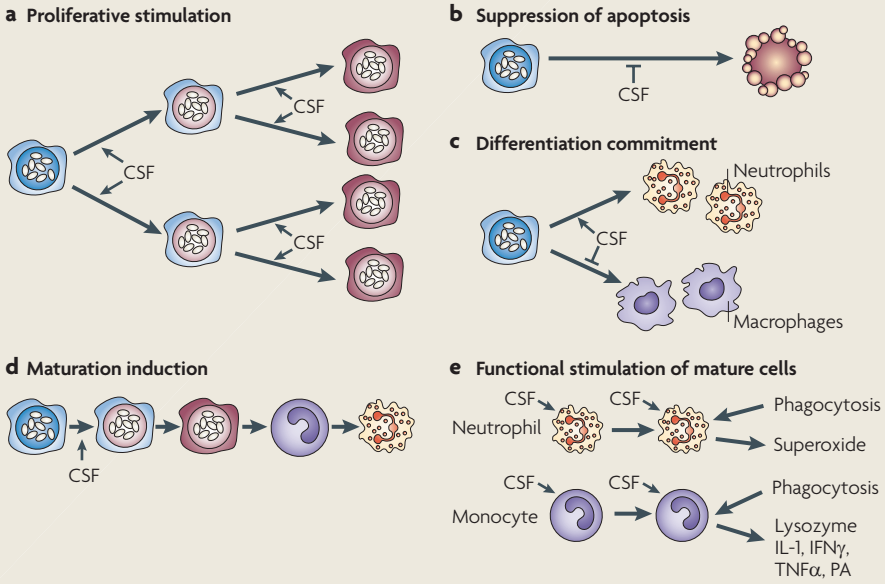
Synergy
Enhanced cellular responses when two or more regulators interact on target cells

Thrombocytopenia
Abnormally low blood platelet levels

Transformed
Usually indicating an irreversible change from normal to neoplastic cells

Box 2 | **The multiple actions of the colony-stimulating factors**

Operating through a single type of membrane receptor on responding cells, the colony-stimulating factors (CSFs) can elicit a surprising range of biological responses. They are necessary to initiate, in a dose-responsive manner, every cell division in responding cells (part **a** of the figure below)[28]. They prevent cell death from apoptosis (part **b** of the figure)[36–39]. They can, arguably, initiate lineage commitment and maturation (parts **c** and **d** of the figure) in appropriate haematopoietic subpopulations[42–44]. Finally, they have powerful effects on the survival and functional activity of mature cells (part **e** of the figure)[47–51]. These pleiotropic effects are made possible by distinct regions of CSF-activated cytoplasmic domains in cognate receptors[52–59]. IFNγ, interferon-γ; IL-1, interleukin 1; PA, plasminogen activator; TNFα, tumour necrosis factor-α.



these mice were more susceptible to leukaemic transformation by the Moloney leukaemia virus[95]. Repopulation of mice for a period of a few months with bone marrow cells that were engineered to produce excess levels of G-CSF or IL-3 did not lead to leukaemia development[87,88]. On this basis, the original simple hypothesis of leukaemia development that led to the search for the CSFs seemed not to be correct.

Despite these negative data, at least *CSF2* and *IL3* can function as oncogenes in haematopoietic cells. In the initial study, *CSF2* cDNA was inserted *in vitro* into FDC-P1 cells, a mouse immortalized haematopoietic cell line. These cells have remained CSF-dependent in culture for the past 25 years and remain non-leukaemic. However, after transfection with *CSF2* cDNA, the FDC-P1 cells were immediately transformed to cells that showed factor-independent growth *in vitro* and behaved as leukaemic cells when transplanted *in vivo*[96].

In a related series of studies, non-leukaemic FDC-P1 cells were injected into pre-irradiated recipients. The injected cells had therefore not been subject to irradiation themselves but were in a host that could better support the survival of these factor-dependent cells. The cells remained dormant for up to 1 year but eventually most mice developed leukaemia[97]. In each case, the leukaemic cells were derived from injected FDC-P1 cells that had acquired an autocrine capacity to produce GM-CSF or IL-3 (REF. 98). Analyses showed that this autocrine capacity to produce CSF was determined by the activating insertion of intracisternal A particles in variable locations upstream of either CSF gene[98]. It is unresolved why extrinsically applied CSF in high concentrations does not transform immortalized cells such as FDC-P1 after decades in culture, whereas autocrine production of the same CSF leads to immediate transformation. However, the drawback to the FDC-P1 cell experiments was that the molecular basis of the original immortalization of this cell line was never clearly established.

In a more informative experiment, normal bone marrow cells were co-transfected with homeobox B8 (*HOXB8*, also known as *HOX-2.4*) and *IL3* cDNAs. HOXB8 modulates self-renewal and, again, there was immediate transformation of the transfected cells to growth factor-independence *in vitro* and to leukaemogenicity *in vivo*[99]. These observations have given rise to the concept that, at least in mice, myeloid leukaemia development requires two types of change: an imbalance of lineage commitment at cell division that favours self-generation or immortalization in an extreme form and the acquisition of a capacity for autocrine growth stimulation.

There is no reason at present to suppose that myeloid leukaemia development in humans differs in principle from that in the mouse. The various leukaemia-associated genes affected by translocation or mutation that have been detected in AML presumably achieve one or the other of the two changes needed in leukaemogenesis[100]. Autocrine production of GM-CSF has been reported in some cases of AML[101]. However, what is of interest in view of the mouse data is that autocrine production of CSF by myeloid leukaemic cells does not seem to be as common in humans as in mice. It has been reported that, in early CML development, transient autocrine production of IL-3 and G-CSF occurs[102], and the presence of activating mutations in the transmembrane region of the extracellular domains of the CSF receptors remain a possibility in some AML populations[103]. More commonly, however, autocrine proliferative stimulation in human AML seems to be achieved by other mechanisms, such as by activation of cellular *MYC* or Ras genes.

A curious outcome of the ability of CSFs to enforce maturation in responding haematopoietic cells is seen in the action of CSFs on some myeloid leukaemia populations. The purification of mouse G-CSF was originally partly monitored using an assay system in which proliferation of WEHI-3B myelomonocytic leukaemia cells was suppressed by G-CSF-enforced maturation[13]. A dramatic example of this type of action was also observed for the mouse GB-2 leukaemia cell line. When these undifferentiated cells were cultured in agar with various CSFs they formed well-differentiated colonies that showed the correct maturation pattern for the CSFs used[42]. Finally, it could be argued that when CSFs are used to stimulate the growth of human CML colonies *in vitro*, which then show normal maturation, CSFs have induced this normal maturation.

Unfortunately, responses to the maturation action of CSFs seem to be a rare feature of human AML populations, and little evidence for a similar therapeutic action of CSFs has been observed in patients that have AML who receive injections of CSF.

Clinical use of CSFs

Results of the first clinical trials of CSFs were published in 1988 and 1989. In general, these tests were performed following chemotherapy. Similar haematopoietic responses to those in the mouse were noted in these preliminary clinical trials on CSFs in so far as these could be monitored in the peripheral blood and bone marrow[104–107]. G-CSF injections elicited clear dose-responsive increases of blood neutrophil levels and GM-CSF elicited lesser responses. Responses were maintained for as long as CSF injections were continued, and from extended studies on children who had abnormally low blood neutrophil levels, no loss of responsiveness was noted after repeated daily G-CSF injections[108,109].

Based on these responses and the minimal toxicity associated with the injection of G-CSF or GM-CSF, the licensing of these agents for clinical research was prompt. For example, G-CSF was registered in the United States in 1991 for use in the prophylaxis of febrile neutropenia in patients with cancer following chemotherapy. Registration of both agents followed in other countries and the indications for clinical use were progressively widened. A less favourable outcome followed trials of M-CSF and IL-3. Intravenous infusion of M-CSF in patients with metastatic cancer was associated with a fall in platelet levels possibly owing to macrophage activation[110]. Similarly, subcutaneous injections of IL-3 in some patients who relapsed with lung cancer after chemotherapy increased neutrophil levels but also led to adverse responses, some of which may have been due to mast cell activation[111]. Neither agent has entered clinical use because of the risk of unacceptable side-effects.

Leaving aside the possible special role that CSFs may have in the biology of myeloid leukaemia, the CSFs have had a major effect on the treatment of cancer in two situations: cytotoxic drug-induced neutropenia and the common need to replace aplastic bone marrow with transplanted haematopoietic cells.

Treating chemotherapy-induced neutropenia. The most common complication of chemotherapeutic treatment of cancer is the development of neutropenia owing to bone marrow damage. Low neutrophil levels are associated with a heightened risk of infection[112] and a substantial proportion (60%) of patients with febrile neutropenia syndrome develops infections. This usually requires hospitalization and intensive antibiotic therapy. Perhaps of more importance for those patients for whom chemotherapy is potentially curative, episodes of neutropenia with or without infections disrupt scheduled chemotherapy, resulting in either dose reduction or loss of treatment cycles.

The initial clinical trials of subcutaneously injected recombinant human G-CSF and GM-CSF were in patients with diverse types of cancer following chemotherapy and the results showed that administration of either agent could increase neutrophil levels even after chemotherapy[104–107]. This resulted in a reduction in the duration and severity of the chemotherapy-induced neutropenia (FIG. 1c). In subsequent trials, it was documented in patients with small-cell lung cancer and non-Hodgkin's lymphoma that the use of G-CSF or GM-CSF reduced episodes of drug reduction and the frequency of infections[113–117]. Analysis showed that use of G-CSF allowed patients with chemotherapy-sensitive cancers, such as non-Hodgkin's lymphoma or early-stage breast cancer, to avoid dose reductions or delays in their chemotherapy, and confirmed that this had an effect on patient survival[118]. GM-CSF was also used effectively to enhance haematopoietic regeneration after bone marrow transplantation[119,120] and was initially licensed for clinical use for this purpose.

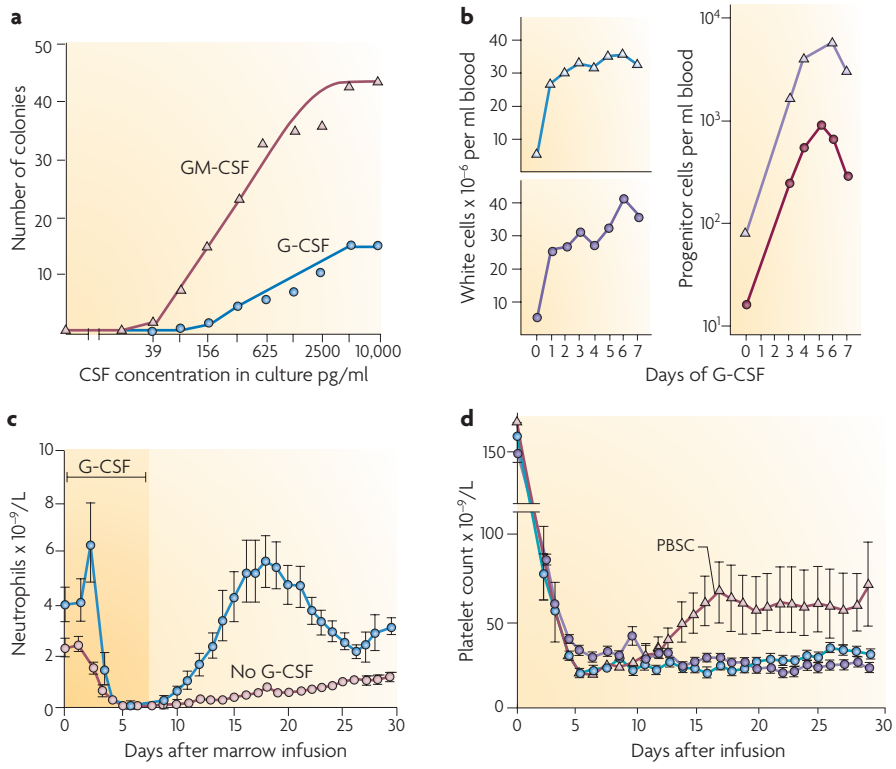


Figure 1 | **The biological actions of the colony-stimulating factors. a** | Colony-stimulating factor (CSF)-stimulated colony formation *in vitro* by lineage-committed progenitor cells in mouse bone marrow, the sigmoid dose response curve indicates heterogeneity in the responsiveness of the progenitor cells. **b** | In humans, injected granulocyte-CSF (G-CSF) not only increases peripheral blood neutrophil levels but also increases peripheral blood progenitor cells 100-fold. Data shown are from two patients injected with 10 μg/kg G-CSF daily for 7 days. **c** | In patients with cancer who have received a transplant, the injection of G-CSF accelerates the recovery of neutrophil levels following chemotherapy, allowing a shorter duration of hospitalization. **d** | Transplantation of CSF-mobilized peripheral blood stem cells augments the rate of recovery of platelet levels following chemotherapy compared with control patients receiving bone marrow transplants. Image in part **a** is modified, with permission, from REF. 28 © (1995) Cambridge University Press. Images in part **b** are modified, with permission, from REF. 158 © (1995) American Society of Hematology. Image in part **c** is modified, with permission, from REF. 159 © (1989) Elsevier. Image in part **d** is modified, with permission, from REF. 136 © (1992) Elsevier.

To date, approximately nine million patients have received G-CSF therapy. These were most often patients with cancer in whom cytotoxic drugs had been used. Overall, meta-analyses of multiple controlled trials involving G-CSF have found that G-CSF reduces febrile neutropenia by 46%, the risk of infection-related mortality by 45% and the risk of early mortality from all causes by 40%[118]. Toxic side-effects of G-CSF have been minor, and the most common is slowly developing bone pain as marrow populations expand[118]. This may be related to the recent report that sensory nerves have receptors for G-CSF and GM-CSF and that both CSFs can sensitize these nerves to mechanical stimuli[121].

Experience with the use of GM-CSF has been similar, if less extensive than with G-CSF. In the context of providing supporting treatment for patients on chemotherapy, clinical attention has properly been focused on responding neutrophil levels because of a landmark study linking low neutrophil levels with infections[112], and in this instance G-CSF has a stronger action than GM-CSF. The subtle differences in biological actions between G-CSF and GM-CSF, such as the special actions of GM-CSF on macrophages and dendritic cells, have not yet had much effect on the manner in which the two agents are used clinically.

A notable advance in the use of CSFs for post-chemotherapy neutropenia was the development of polyethylene glycol-conjugated G-CSF (pegylated G-CSF; pegfilgrastim), which was approved for clinical use in 2002 following Phase II trials on patients with cancer receiving chemotherapy[122,123]. The biological actions of this modified CSF are similar to those of G-CSF, but the larger size of the pegylated molecule prevents renal clearance and greatly increases the lifespan of the molecule — a single injection of pegylated G-CSF is equivalent to a series of daily injections of G-CSF. Studies have shown the efficacy of pegylated CSF in allowing full-dose chemotherapy, particularly in elderly patients who would otherwise have been restricted to less toxic, mild to moderate chemotherapy[118].

Currently, international guidelines recommend the use of G-CSF as primary prophylaxis when there is an increased risk of febrile neutropenia of greater than or equal to 20%, although the broader use of prophylactic CSF has been suggested[118,124]. How extensively a non-toxic agent is used is partly based on economic criteria and, when the costs of CSFs are reduced by the introduction of generic CSFs, the occasions in which CSFs may be used should increase.

From a biological point of view, current clinical practice is probably suboptimal because it has made no use of the powerful synergy to be obtained by combining CSFs or combining CSFs with other agents. In addition, insufficient use has been made of the facts that CSFs function best when used prophylactically before infections initiate and on bone marrow that has reasonable cellularity.

> “CSFs have emerged as key regulators of major haematopoietic lineages and two have been in clinical use for two decades”

As an aside and for completeness, there are of course less common types of patients who do not have cancer, such as those with chronic neutropenia or cyclic neutropenia, for whom the use of G-CSF has been highly effective in preventing infections and G-CSF has been administered for years without loss of activity or major adverse long-term effects[125,126].

It is of interest how the development of the CSFs paralleled that of EPO, the corresponding regulator of erythroid cell populations. The existence of EPO was recognized long before the CSFs but, even so, EPO was purified in 1977 (REF. 127), cloned in 1985 (REF. 128) and was approved for clinical use in 1989. It is now used routinely in patients with anaemia associated with chronic renal disease and often in patients who have cancer and anaemia — in both situations to reduce the number of blood transfusions and increase survival and quality of life[129,130]. In both types of patient, to avoid cardiovascular complications, EPO-induced rises in haemoglobin levels need to be restricted to below 120g/l.

Haematopoietic transplantation. During the first clinical trials of G-CSF in patients with cancer in 1988, an unexpected observation was made — the patients developed a 100-fold rise in the frequency of colony-forming progenitor cells in the peripheral blood[131] (FIG. 1b). Rises in haematopoietic progenitor cell numbers were also noted in subjects injected with GM-CSF[132,133]. Although slightly delayed compared with the rises in mature neutrophil levels, these rises were of such magnitude that it became an intriguing possibility that CSF-induced cells in the peripheral blood might be used for transplantation in place of harvested bone marrow cells.

Subsequent studies in mice in 1990 showed that G-CSF could also elicit rises in haematopoietic stem cells in the blood[134]. With this supporting information, clinical trials were initiated using peripheral blood cells harvested after injections of GM-CSF or G-CSF. Both types of peripheral blood stem cells (PBSCs) led to successful haematopoietic engraftment[135,136].

The PBSCs generated were found to result in more rapid rates of restoration of peripheral blood neutrophil levels than those achieved by harvested bone marrow cells and equalled the recovery of cells in patients who received bone marrow plus CSF[137]. In addition, CSF-induced PBSCs unexpectedly allowed a more rapid recovery of platelet levels[136,138] (FIG. 1d). Thrombocytopenia, which requires treatment with platelet transfusions, is an important reason for continued hospitalization of patients with cancer with myelosuppression following chemotherapy. It is now accepted that the superiority of CSF-elicited PBSCs in transplantation is probably owing to the ability to harvest higher numbers of stem cells and CD34$^+$ progenitor cells than by routine bone marrow aspiration. Chemotherapy itself can increase the numbers of PBSCs[139], and higher levels of harvested PBSCs can be obtained by combining CSF with chemotherapy. However, CSF alone usually achieves satisfactory yields of PBSCs, and chemotherapy cannot be used when normal donors are providing PBSCs for allografting to patients.

The high cell yields possible after daily injection of CSF or a single injection of pegylated CSF are of great importance when low- or medium-intensity chemotherapy is used to treat elderly patients. High numbers of haematopoietic progenitor and stem cells are required in these patients for adequate engraftment. This is not because of any failure to empty bone marrow niches in the recipient of less severe chemotherapy. Populations of grafted cells that have stable chimaerism can be achieved in normal mice simply by increasing the numbers of transplanted cells so that they comprise a substantial fraction

of the resident cells in the recipient[140]. This same principle seems to operate in elderly patients receiving low-dose chemotherapy when the high numbers of harvested PBSCs allow adequate engraftment.

CSF-mobilized PBSCs have now become the dominant cell populations used in transplantations to patients with cancer and other patients. Particularly for normal donors of PBSCs, the safety of G-CSF and GM-CSF is a matter of importance. Extensive clinical experience has shown that CSF induction is a safe procedure without any immediate or long-term consequences[141].

Immunotherapy. Dendritic cells are key cellular components of immune responses because of their capacity to capture, process and present antigens to initiate responses in T lymphocytes. GM-CSF was observed to be a major regulator of dendritic cell development *in vitro*[142–145], and this has led to the study of the positive influence of GM-CSF on immune responses. With the increasing availability of tumour-specific peptides, there has been much interest in the possibility that GM-CSF could enhance specific immune responses against tumour cells. The cancers most frequently considered in this context are melanomas and cancers of the kidney, lung and prostate — cancers for which some evidence exists that host responses can occasionally have substantial anti-tumour effects. GM-CSF has variously been co-injected with tumour peptides, injected as a GM-CSF–peptide complex or transfected into sterilized autologous or similar tumour cells using retroviral or adenoviral vectors[146]. GM-CSF proved to be the most potent of ten candidate gene products in tests to detect enhanced responses elicited by transfected tumour cells[147]. Evidence of enhanced local immune responses in tumours has been obtained in a proportion of patients[148–154]. To date, positive clinical responses have been restricted to a small subset of patients who have been injected with GM-CSF, and it is unclear whether these responses have been superior to those obtained using tumour peptides alone[146,155]. These are ongoing studies and it is too early to decide whether the use of GM-CSF in immunization strategies will prove of clinical value.

Conclusions

It has been a long journey since the first agar cultures of bone marrow cells in 1966. The CSFs have emerged as key regulators of major haematopoietic lineages and two have been in clinical use for two decades to stimulate neutrophil and macrophage production and function, particularly in patients with cancer. The use of CSFs to elicit peripheral blood stem cells has revolutionized haematopoietic transplantation, making it simpler, more efficient and more widely applicable in the clinic. Despite this progress, it is still early days in the clinical exploitation of the CSFs to further manipulate haematopoiesis to improve the management of patients with cancer.

Although autocrine production of CSF can be involved in one of the steps in the development of myeloid leukaemia, the maturation-inducing effects of CSFs can conversely suppress some myeloid leukaemia populations. The clinical application of this complex biology of CSFs again awaits future developments.

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doi:10.1038/nrc2843

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Acknowledgements
The author is indebted to grant support from the Cancer Council of Victoria; the National Institutes of Health, Bethesda grant number CA22556; and the National Health and Medical Research Council, Canberra, program grant number 461219.

Competing interests statement
The authors declare competing financial interests; see Web version for details.

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