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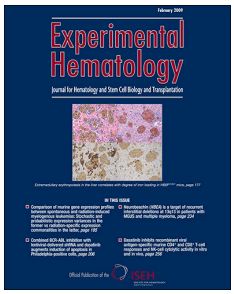

In vivo at last. Demonstrating the biological credentials and clinical potential of GM-CSF

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In vivo at last. Demonstrating the biological credentials and clinical potential of GM-CSF.

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Abstract

The pioneering contribution of Professor Donald Metcalf, who passed away in 2014, to the discovery and characterisation of the colony stimulating factors (CSFs) is exemplified by a seminal contribution to *Experimental Haematology* by Metcalf and colleagues that detailed the *in vivo* actions of the newly available recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) in 1987. The results described in this publication promoted GM-CSF actions from fascinating *in vitro* laboratory phenomena to the recognition that this cytokine was a genuine *in vivo* regulator of blood cell production and function and provided significant impetus for the clinical development of GM-CSF.

The fact that soluble regulators, the cytokines, growth factors and colony stimulating factors, continually shape the blood-forming system by stimulating hematopoietic cell survival, proliferation, maturation and functional activity, is axiomatic in today's medical research and clinical practice. As recently as 30 years ago, the relevance of such a system of blood cell regulation was considerably less obvious. The colony stimulating factors (CSFs): macrophage (M)-CSF, granulocyte-macrophage (GM)-CSF, granulocyte (G)-CSF and interleukin-3 (IL-3) or multi-CSF, were among the first soluble blood cell regulators to be discovered and fully characterised. In this series focusing on influential contributions to *Experimental Hematology*, this article acknowledges Professor Donald Metcalf, who passed away in late 2014. Metcalf made a pioneering 60-year contribution to the understanding of molecular and cellular hematology. This article focuses on a seminal contribution to *Experimental Hematology* published by Metcalf and colleagues in 1987 [1], a paper that contributed profoundly to defining the *in vivo* actions of the newly available recombinant GM-CSF. The context and challenges faced by Metcalf and his colleagues and collaborators leading up to this publication are briefly described and the significance and influence of its results are discussed.

For Metcalf, the path to the discovery and characterisation of the CSFs began in the late 1950s. Newly appointed as the Carden Fellow of the Anti-Cancer Council of Victoria at the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia, a Fellowship he held for 60 years, he began working on leukemia, with an initial focus on the thymus and its apparent role in certain leukemias, including investigation of factors capable of inducing lymphocytosis [2]. Further influenced during a post-doctoral stint at Harvard University by Jacob Furth's studies implicating hormonal imbalance in endocrine tumors, Metcalf began to seriously consider whether similar regulators might exist for controlling blood cell production and function and that such regulators might also potentially contribute to leukemia. In 1964, Ray Bradley, who was collaborating with Metcalf investigating the behavior of thymic grafts, showed Metcalf an experiment in which he had

attempted to grow AKR thymic lymphoma cells in agar cultures in the presence of bone marrow 'feeder' cells. Colonies of cells had grown, but within the bone marrow underlayer rather than in the layer containing the thymoma cells. Further investigation revealed that growth of bone marrow cell colonies was absolutely dependent on co-cultured cells and Metcalf and Bradley surmised that a soluble factor released from the co-cultured cells was stimulating bone marrow cell proliferation and thus colony formation [3], and as early as 1966 they were able to replace the feeder layers with cell-free stimuli such as leukemic mouse serum. At the same time, Pluznik and Sachs were growing colonies from mouse bone marrow using a similar clonal culture technique [4]. The technique for identifying the colony-stimulating factors was thus born, heralding a new era of cellular and, ultimately, molecular hematology.

The initial observations using the new colony assay were quickly verified and it became clear that the notion of a soluble tissue-derived substance that promoted the growth of granulocytes and macrophages was indeed robust. While at first it was assumed that the activity responsible for colony formation in clonal cultures was due to a single agent, during the 1980s several distinct factors were identified that were capable of stimulating granulocyte and/or macrophage colony formation, each with distinguishable biochemical properties and biological specificities. These discoveries not only resulted in a confusing initial nomenclature (Table 1), but also added a very significant degree of difficulty to the task of characterising and defining the separate activities in complex tissue extracts or cell-conditioned media. The prospect of purifying the individual factors responsible for the colony stimulating activities appeared to be an imposing task. Given the very small amounts of CSFs available from tissue-derived sources and the limitations of available techniques at the time, the ultimate purification from mouse lung-conditioned medium of what was to become known as GM-CSF, achieved by Metcalf's colleagues in 1977 [5, 6], required extraordinary persistence and dedication over a period of more than a decade.

The miniscule amounts of the CSFs that could be purified from cellular and tissue sources had frustrated the examination of their *in vivo* activities. By the mid 1980s, a burgeoning literature had defined in relative detail the *in vitro* properties of the CSFs, but an understanding of their *in vivo* physiological and pathological roles – and indeed the prospects of any potential clinical utility – demanded *in vivo* analyses. It became clear that these would require purification of sufficient CSF to allow amino acid sequencing to inform design of nucleic acid probes for the cloning and ultimate expression of recombinant protein. To achieve this for GM-CSF, Metcalf and his colleagues enlisted the assistance of collaborators at the Protein Chemistry Division of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) of Australia and the N-terminal amino acid sequence was determined at the California Institute of Technology [6]. In collaboration with the newly established Ludwig Institute for Cancer Research in Melbourne, this amino acid sequence was used to design and synthesise degenerate oligonucleotide probes for isolation of murine GM-CSF cDNA clones [7].

Despite the overwhelming evidence *in vitro*, some anxiety regarding the *in vivo* activity and physiological significance of the CSFs lingered. Pessimistic views in the field posited that hematopoiesis was so highly self-regulating that any effects of administration of a single stimulatory factor would be dampened by homeostatic controls. Others believed that it would not be feasible to achieve sufficiently elevated concentrations above the normal levels of the CSFs, particularly in local environments such as the densely populated bone marrow, that would be sufficient to stimulate additional cellular proliferation, or that if responses could be achieved they would be modest and transient. Until the activities of the CSFs could be tested in the whole animal, the actions of these regulators formally remained a laboratory phenomenon.

The *in vivo* actions of purified recombinant GM-CSF (rGM-CSF) were reported by Metcalf and colleagues in *Experimental Hematology* in 1987 [1], a report among a pioneering set of

publications first exploring the *in vivo* activity of purified recombinant murine or human GM-CSF in mice and primates [8-10]. In the Metcalf *et al.* study, the recombinant mouse protein was expressed in *E.coli* and purified by Biogen in Geneva, with mice injected and analysed by Metcalf and colleagues in Melbourne. In this initial study a single injection of rGM-CSF proved sufficient to yield detectable circulating GM-CSF activity that displayed a half-life of around 30 minutes. Mice injected intraperitoneally with a course of rGM-CSF over several days displayed minor changes in blood cell numbers with a modest increase in circulating neutrophils evident. However, significant increases in the numbers of macrophages, eosinophils and neutrophils were observed in the peritoneum; in some animals this was up to 100-fold more than usually observed in uninjected mice. The peritoneal macrophages in rGM-CSF-treated mice were larger, more highly vacuolated and more mitotically active than resident cells in untreated controls. The GM-CSF-elicited macrophages also exhibited a dose-related increase in phagocytic activity. No changes were observed in the numbers of lymphocytes. An almost 50% increase in spleen weight at higher rGM-CSF doses was accompanied by a modest increase in the proportion of monocytes and the numbers of megakaryocytes in this organ although there were no consistent changes in the relative proportions of neutrophils or eosinophils. The numbers of non-erythroid clonogenic progenitor cells in the spleens of mice treated with rGM-CSF were increased up to 4-fold at higher doses but the relative frequencies of progenitor cells committed to granulocyte, macrophage or eosinophil production were unaltered relative to controls. In the bone marrow, a significant decrease in overall cellularity was evident upon treatment with rGM-CSF and was specifically associated with reduced numbers of lymphocytes and nucleated erythroid cells; the numbers of granulocytes, monocytes and eosinophils remained unchanged. Numbers of non-erythroid bone marrow progenitor cells were also reduced with no effect on the relative proportions of granulocyte, macrophage or eosinophil colony-forming cells. Infiltrating neutrophils were evident in the lungs and livers of rGM-CSF-treated mice, and these were accompanied by increased numbers of macrophages and occasionally eosinophils and megakaryocytes in the latter organ.

It took 20 years from the time Bradley and Metcalf inferred the existence of CSFs from their first clonogenic bone marrow cultures for Metcalf and his colleagues and collaborators to complete the characterization, purification, cloning and production of recombinant GM-CSF protein that permitted testing of the actions of this regulator *in vivo*. While Metcalf and colleagues' paper in *Experimental Hematology* in 1987 [1] and the contemporaneous publications from others [8-10] were followed by many subsequent *in vivo* analyses that refined the understanding of the actions of GM-CSF, these first results in 1987 were both exciting and profound. First and foremost, the results of these studies eliminated any doubt that administration of recombinant GM-CSF could stimulate hematopoietic production in the whole animal, and the cells affected *in vivo*, primarily granulocytes, monocytes/macrophages, eosinophils and to some extent megakaryocytes, reflected the activities of GM-CSF in clonogenic assays. Interestingly, the most dramatic effects of rGM-CSF injection were observed at the local site of injection within the peritoneum and the observation that rGM-CSF stimulated phagocytic activity in macrophages was consistent with previous studies *in vitro* that showed that stimulation of myeloid cells by GM-CSF increased their effector functions [11-13] and presaged the intensive investigation of the roles of GM-CSF in functional activation of hematopoietic cells.

Two issues of *Experimental Hematology* later, Metcalf and colleagues published results of an assessment of the *in vivo* actions of recombinant IL-3 administration [14]. When considered in combination with studies published previously [15-17], these observations demonstrated that IL-3-elicited increases in non-lymphoid cells and their progenitors in the blood and/or hematopoietic tissues, with a notable effect on mast cells. This was soon followed by the demonstration that injection of G-CSF could induce an extraordinary increase in circulating neutrophils [18, 19]. Together with the delineation of the *in vivo* actions of GM-CSF discussed above, these pioneering studies confirmed the close similarity between the *in vitro* and *in vivo* actions of the CSFs,

established that the sustained elevation in myeloid cells numbers was achievable at appropriate doses of a CSF, and began to identify the key differences in the biological actions of these regulators within the whole animal. These studies set the stage for the intensive evaluation of the physiological roles of CSFs, the contributions of these cytokines to diseases of the blood and other organs, and their potential for clinical application.

The results of these first studies of GM-CSF activity *in vivo* began to define the effects of administration of supra-physiological doses of recombinant protein. However, Metcalf and colleagues were also encouraged to conclude that their data made it likely that GM-CSF would "function *in vivo* as a genuine regulator of granulocyte-macrophage populations" [1]. Subsequent studies have proven this to be the case, including definitive analyses of the indispensible functions of GM-CSF in mice. When the gene encoding GM-CSF is ablated, mice lacking GM-CSF develop normally and display no major hematopoietic abnormalities during the first few months of life, with production of normal numbers of myeloid cells. However, in the absence of GM-CSF, the mice invariably accumulate surfactant lipids and proteins within the lung alveolar spaces, accompanied by mixed hematopoietic infiltration, the presence of large phagocytic macrophages and often they develop subclinical fungal or bacterial infections [20, 21]. This pathology is typical of pulmonary alveolar proteinosis (PAP) in humans and subsequent studies demonstrated that most cases of PAP were associated with the production of autoantibodies to GM-CSF [22]. Mice lacking GM-CSF also demonstrate increased sensitivity to some infectious agents [23-26]. Together these data suggest that while GM-CSF plays a limited role in steady-state hematopoiesis, it is required for normal lung physiology and adequate host response to infection, probably via its effects on hematopoietic effector cell function.

During the years the CSFs were being identified, purified and then subsequently cloned and assessed for *in vitro* activity, the possibility that these regulators might have some clinical utility

became increasingly likely. Although a key driver of their research, Metcalf and colleagues were careful not to emphasise the potential clinical uses of CSFs until the data from *in vivo* studies began to emerge. In the *Experimental Hematology* paper under discussion here, they were now sufficiently confident to propose that "In the clinical context, the administration of rGM-CSF can be expected to result in useful stimulation of hemopoietic populations in patients with subnormal hemopoiesis, e.g., following cytotoxic therapy, and in patients with refractory infections." [1].

Although it took over two decades between the discovery of colony stimulating activity using clonogenic bone marrow assays and the demonstration of *in vivo* activity of these regulators, there was little subsequent delay prior to the clinical evaluation of the CSFs. Much of the initial clinical appraisal of GM-CSF focused on its effects in cancer patients undergoing cytotoxic therapy. It was hoped that CSF might reduce the impact of bone marrow damage and reduce the decreases in white cell counts and the consequential life-threatening infections. Although careful attention to dose was required, with higher doses associated with inflammation-like symptoms, GM-CSF did indeed prove effective in reducing the hematological side-effects of cancer therapy and is now approved widely for therapeutic use in the clinic. Most notably, GM-CSF shortens the duration of chemotherapy-induced neutropenia and reduces life-threatening infections in patients with leukemia, especially in older patients. GM-CSF also enhances myeloid recovery following bone marrow or peripheral blood transplantation and increases the numbers of peripheral blood stem cells [27, 28].

In addition to its myelopoietic activity, the immune-stimulating effects of GM-CSF and its application for management of infectious diseases continue to be evaluated [29]. Moreover, the effects of GM-CSF on cytotoxic T-cells and/or dendritic cells are being explored for the treatment of solid tumors such as renal cell carcinoma and lung cancer, and as a potential immunostimulatory enhancer of cell-mediated anti-tumor vaccines [28, 30]. Finally, the proinflammatory activities of GM-CSF are implicated in the pathology of several autoimmune and inflammatory diseases such as

arthritis, asthma and multiple sclerosis, and clinical trials of specific inhibitors of GM-CSF or its receptor are currently underway with early promise of improved future management of these diseases [31, 32].

GM-CSF is now used routinely in clinical practice and ongoing research continues to uncover important actions and potential additional clinical uses. The 1987 publication in *Experimental Hematology* highlighted here is a fitting example of the broader and profound contributions and influence of Professor Donald Metcalf and his colleagues and collaborators to research in cellular and molecular hematology. The formal demonstration of the activity of GM-CSF *in vivo* was the culmination of decades of discovery and innovation, was an indispensible contribution to the current understanding and ongoing clinical appraisal of cytokine biology, and was the forerunner of further profound research contributions from Metcalf and colleagues to refining the clinical applications of the CSFs and to discovery of their receptors and signalling cascades. The results described in this publication were among the first that promoted GM-CSF actions from fascinating *in vitro* laboratory phenomena to the recognition that this cytokine was a genuine regulator of blood cell production and function. The data significantly helped to provide the impetus for the development of GM-CSF as a drug that has helped myriad patients world wide.

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Table 1. The colony stimulating factors

CSF	Selected alternative names	Gene nomenclature	First *in vivo* testing in mice [33]	First Clinical Trials [33]
Macrophage colony-stimulating factor (M-CSF)	CSF-1, Macrophage growth factor (MGF), macrophage and granulocyte inducer IM (MGI-IM)	*csf1* (mouse), *CSF1* (human)	1988	1992
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	CSF-2, macrophage and granulocyte inducer type 1, granulocyte-macrophage (MGI-1GM), CSFα, pluripoietin α	*csf2* (mouse), *CSF2* (human)	1987	1987
Granulocyte colony-stimulating factor (G-CSF)	CSF-3, macrophage and granulocyte inducer type 1, granulocyte (MGI-1G), pluripoietin	*csf3* (mouse), *CSF3* (human)	1986	1988
Interleukin-3 (IL-3)	Multi-lineage CSF, Burst-promoting activity (BPA), Persisting (P) cell stimulating factor (PSF), Haemopoietic cell growth factor (HCGF), Thy-1-stimulating activity, Histamine-producing cell stimulating activity, CFUs-stimulating activity, Stem cell activating factor (SAF), Pan-specific haemopoietin	*il3* (mouse), *IL3* (human)	1986	1990

Figure 1. Professor Donald Metcalf assessing agar cultures *circa* 1980s (*Photograph courtesy of The Walter and Eliza Hall Institute of Medical Research*).