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Original Paper

GM-CSF and Stimulation of Monocyte/Macrophage Function *In Vivo* Relevance and *In Vitro* Observations

M.A. Williams, S.M. Kelsey and A.C. Newland

Department of Haematology, St Bartholomew's and the Royal London Hospital School of Medicine and Dentistry, Queen Mary and Westfield College, University of London, Whitechapel, London El 2AD, U.K.

INTRODUCTION

UNTIL RECENTLY, the physiological mechanisms that regulate humoral and cellular immunity have remained poorly defined. However, new experimental approaches have revealed greater insight into the regulation of the immune system. Accompanying this knowledge has been an accelerated interest in new therapeutic strategies aimed at treating infection and malignancy. Strategies that aim to treat diseases by modification of host immunity are termed immunotherapy. Immunotherapeutic approaches can intervene in the disease processes associated with systemic microbial disease and against malignancy. The goal of immunotherapy is to correct or augment a suppressed immune system. It is intended that this will contribute to the eradication of the underlying disease. We are particularly interested in the monocyte system since they constitute an important component of host immunity and are effective cellular weapons against both micro-organisms and neoplasia. In this paper the function of the monocyte system will be discussed in the context of *in vitro* and *ex vivo* observations conducted in our laboratory and in the context of their potential immunotherapeutic value against cancer.

MODULATION OF MONOCYTE ADHESION MOLECULE EXPRESSION AND RESPIRATORY BURST ACTIVITY

The monocyte system serves a key role in immunity. By a process regulated by locally synthesised cytokines and chemokines, monocytes adhere to the vascular endothelium and transmigrate to the surrounding tissues. Newly emigrated monocytes migrate to inflammatory foci where they become activated. Under resting conditions the monocyte system exists as a pool of non-activated cells thus avoiding autoimmune dysfunction. However, during an inflammatory response, cytokines secreted by, for example, activated T cells or secreted components of micro-organisms such as lipopolysaccharide (LPS) prime the monocyte to respond to further activation where monocyte functions are up-regulated.

We have shown that administration of rhGM-CSF to patients after chemotherapy augmented the membrane-expression of β2-integrin adhesion molecules as compared with patients given chemotherapy alone at haemopoietic regeneration [1]. This effect persisted for several weeks after rhGM-CSF therapy had ceased. In addition, administration of rhGM-CSF allowed cellular adhesion molecules to respond normally to LPS [1]. rhGM-CSF therapy restored normal functional responsiveness of monocytes and reversed the anergic or refractory status of monocytes that existed in patients given chemotherapy alone. We have also observed monocyte anergy in patients with septic shock, a condition termed immunoparalysis [2]. In this study we clearly showed that monocytes stimulated with rhGM-CSF *ex vivo* responded normally to subsequent stimulation with LPS.

GM-CSF therapy also modulates monocyte respiratory burst activity [1]. Monocytes secrete reactive oxygen species (H_2O_2, O_2^- and OH^-) that are cytotoxic to micro-organisms and tumour cells. Activation of the respiratory burst by rhGM-CSF may be of increased importance in patients with neutropenia. Therapy with rhGM-CSF given after chemotherapy augmented the secretion of H_2O_2, an effect which persisted for several weeks after the cessation of rhGM-CSF therapy [1]. Under *in vitro* conditions that mimicked gram-negative (LPS-induced) and gram-positive (opsonised *Staphylococcus-aureus*-induced) sepsis, an enhanced release of H_2O_2 by monocytes was observed [1].

THE TUMORICIDAL ROLE OF THE MONOCYTE

The monocyte system is the major inducible source of TNF, which is secreted in response to endogenous soluble factors such as LPS. The potentially toxic consequences of uncontrolled TNF secretion may be regulated within the host in a number of ways. For example, the serum half-life of TNF is very short and it is rapidly excreted. In addition, soluble counterparts of TNF receptors such as TNFRp75 (TNF binding proteins) are secreted from activated cells in response to high concentrations of soluble TNF and neutralise its bioactivity [3]. The TNF-TNFRp75 complex is rapidly excreted. In contrast, TNFRp75 may augment the activity of TNF when present in low concentrations by prolonging the biological half-life of TNF.

Correspondence to M.A. Williams, e-mail: m.williams@mds.qmw.ac.uk

THE SYNTHESIS AND IMMUNOLOGICAL FUNCTION OF MONOCYTE-DERIVED TNF

TNF was initially described as a soluble factor that mediated tumour destruction and is the predominant mechanism whereby monocytes kill susceptible tumour cells [4]. TNF is also a multifunctional cytokine that also modulates inflammatory and immunological processes important in host defence. TNF belongs to a family of related polypeptides that include Fas ligand, lymphotoxin, CD27 ligand, CD30 ligand, CD40 ligand and TNF-related apoptosis-inducing ligand or TRAIL [5].

The cytotoxic activity of TNF may be mediated via a transmembrane-associated glycoprotein, pro-TNF, of 26 kD or via mature trimeric TNF of 17 kD that is secreted following membrane metalloproteinease-mediated cleavage of pro-TNF [4, 6]. TNF mediates its cytolytic action by high-affinity binding to TNF receptors (TNFR) that are expressed on the target cell. Two TNF receptors have been identified with molecular weights of 55 kD (TNFRp55) and 75 kD (TNFRp75). Membrane-bound TNF has been found to be more active than soluble TNF in activating TNFR-p75 [6], suggesting that secretion of TNF (and other members of the TNF family) may attenuate the local cell-to-cell activity of membrane TNF. TNF-induced death signalling pathways that involve TNFR and lead to programmed cell death or apoptosis of the target cell have been identified and include other members of the TNF family including the participation of the Fas antigen system [5].

We have shown that rhGM-CSF modulates the expression and secretion of monocyte TNF as well as TNFRp55 and TNFRp75 after chemotherapy [7]. Membrane expression of TNF (Figure 1a) and secretion of immunoreactive TNF (Figure 1b) was increased at haemopoietic regeneration as compared with that of patients who had received chemotherapy alone [7]. This effect persisted for several weeks after cessation of rhGM-CSF therapy and concordant with our observations above. In patients given rhGM-CSF therapy responsiveness of monocytes to LPS was restored, which appeared to be diminished after chemotherapy alone [7]. Expression and secretion of TNFRp55 and TNFRp75 by monocytes were augmented by rhGM-CSF therapy in association with the increase in TNF protein. We propose that rhGM-CSF administration after chemotherapy restores the normal responsiveness of monocytes to secondary stimulation by LPS and primes monocytes to respond to LPS with increased expression and secretion of soluble TNF receptors [7]. These *in vivo* effects of rhGM-CSF in patients with non-myeloid malignancy were concordant with the *in vitro* effects of rhGM-CSF on the expression of TNF and TNFRp55 and TNFRp75 in monocytes.

THE ANTITUMOUR FUNCTION OF THE MONOCYTE SYSTEM

The tumoricidal function of monocytes is manifold. It may serve an accessory role in recruiting and presenting antigen to T-lymphocytes, or it may serve an effector role. The antitumour role of monocytes is not major histocompatibility antigen- (MHC)-restricted and may manifest itself in two ways. First, as an antibody-dependent cellular cytotoxicity (ADCC) mechanism, where the monocyte recognises specific antibody-coated tumour antigens that activate the phagocytic mechanisms of the monocyte, Secondly, as an antibody-independent cellular cytotoxicity, where intimate contact with the tumour is required for effective cytotoxicity. In this second mechanism, the monocyte is activated and its secretory and effector functions are up-regulated.

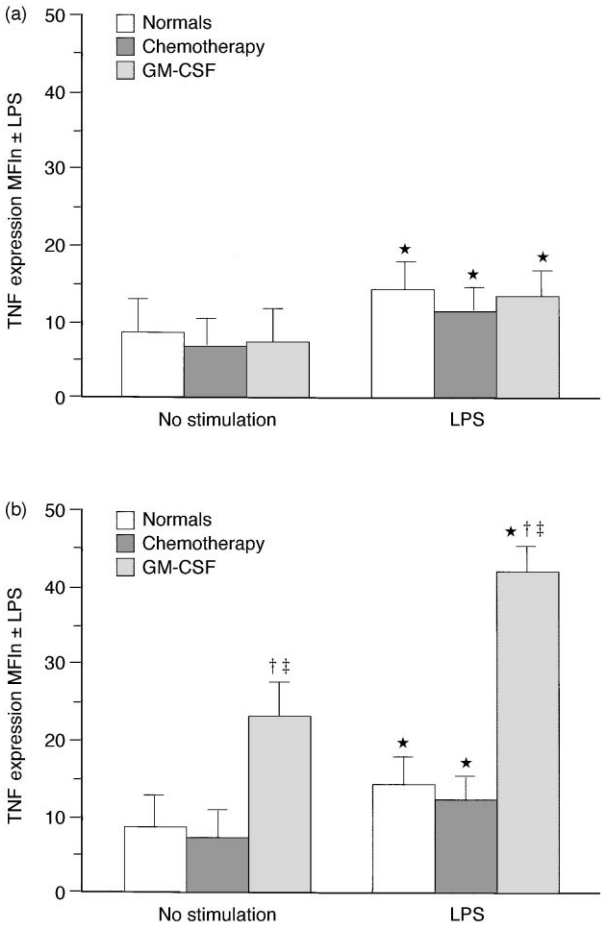

Figure 1. The effect of rhGM-CSF upon monocyte expression of membrane-associated TNF as enumerated before chemotherapy (a) and at regeneration (b). 20 haematologically normal patients in remission or in plateau phase were treated with a single intravenous (i.v.) injection of cyclosphosphamide at 4 g/m² followed by 5 μg/kg rhGM-CSF as a daily subcutaneous (s.c.) injection to mobilise PBPC. 15 patients with CGL in chronic phase and 6 patients with myeloma in plateau phase were recruited as a control group. Patients with CGL received idarubicin 20 mg/m² and cytarabine 1 g/m² prior to PBPC harvest and patients with myeloma received a single i.v. injection of cyclosphosphamide at 4 g/m². Control group patients then received 263 μg rhG-CSF as a daily s.c. injection to mobilise PBPC. 34 age- and sex-matched control subjects who did not receive growth factor or chemotherapy were studied simultaneously. Resting levels of TNF are shown on the left-hand-side of the graph and LPS-stimulated monocytes on the right-hand-side. Normal subjects, patients treated with chemotherapy alone and patients treated with rhGM-CSF exhibited relatively low levels of TNF prior to chemotherapy (a). LPS stimulation up-regulated TNF expression in all patients prior to chemotherapy (a). Augmentation of TNF expression was observed in patients treated with rhGM-CSF at regeneration (b) and even several weeks after cessation of rhGM-CSF (data not shown). The data are expressed as the MFIn. *LPS stimulation up-regulates TNF over resting cells at $P<0.01$. †TNF expression greater than patients treated with chemotherapy alone ($P<0.01$, by MANOVA). ‡TNF expression greater than that observed prior to chemotherapy ($P<0.01$, by MANOVA)

Although monocytes also cooperate with T- and B-lymphocytes in the cell-mediated immune response against malignancy they may also act as the primary tumoricidal effector cell and modulating their function is a desirable goal. Monocyte-mediated cytotoxicity against susceptible tumour targets is thought to occur by induction of apoptosis. Conjugation-dependent killing of tumours may represent an alternative or complementary mechanism to soluble TNF-mediated cytotoxicity. Tumour targets resistant to secreted TNF are effectively killed under conditions facilitating monocyte-tumour conjugation [8]. This mode of monocyte-mediated killing is predominantly TNF-dependent as it can be almost completely abolished by specific neutralising antisera to TNF.

GM-CSF ACTIVATES THE TUMORICIDAL FUNCTION OF MONOCYTES

We have shown that rhGM-CSF augments the anti-leukaemic function of monocytes *ex vivo* [4]. We used the human leukaemic cell-lines (K562, U937 and KG-1) as models of human leukaemia as they exhibit differential sensitivity to cell-mediated or TNF-mediated cytotoxicity *in vitro* (Table 1). Our *in vitro* observations determined that monocyte tumoricidal activity was augmented by rhGM-CSF whereas rhG-CSF was without effect (Table 1). Moreover, cell-mediated killing of leukaemia blasts was augmented by rhGM-CSF administration to patients following chemotherapy [4] (Figure 2). This effect persisted for up to 4 weeks after cessation of rhGM-CSF therapy. rhGM-CSF administration also increased the anti-leukaemic activity of monocytes against targets that were resistant to secreted TNF, probably via a transmembrane TNF-dependent mechanism requiring cell-to-cell contact [4]. Thus, rhGM-CSF augments the tumoricidal properties of the monocyte following myelosuppressive chemotherapy and the killing mechanism is direct and not mediated by an ADCC-type mechanism.

It was unclear why the effect of GM-CSF therapy on monocyte function persisted for several weeks after cessation of growth factor therapy. The biological half-life of GM-CSF after subcutaneous administration is 10–14 h. Three possible explanations may be put forward. Firstly, the persistence of activated monocytes in the blood for several weeks after GM-CSF exposure in the absence of a well-defined inflammatory site for tissue migration.

Secondly, the presence of inducible nitric oxide which alters the haemodynamic behaviour of leucocytes and their interactions with the endothelia, thus abrogating transmigration of monocytes to the surrounding tissues [9]. A third mechanism may involve the differential signal transduction effects of TNF. It has been shown that transmembrane TNF predominates over soluble TNF in activating TNFRp75 for various functions including T cell activation and GM-CSF production [10]. Activation of TNFRp75 by transmembrane TNF leads to qualitatively different TNF responses, such as sensitising tumour cells to TNF-mediated cytotoxicity. Thus in tumour targets that are resistant to soluble TNF-mediated cytotoxicity, the same targets are sensitive to monocyte transmembrane TNF-mediated killing.

Monocytes also secrete enhanced levels of GM-CSF when patients are treated with rhGM-CSF following autologous bone marrow transplantation [11]. Although this requires empirical demonstration *in vivo*, it offers a unique mechanism for our observations of a sustained autocrine-mediated activation of monocytes after the cessation of rhGM-CSF therapy.

IN VIVO RELEVANCE OF MONOCYTE ACTIVATION BY GM-CSF

Other studies support a role for GM-CSF in augmenting the tumoricidal activity of monocytes *in vivo* [12–15]. Wiltchke and colleagues studied the effect of GM-CSF or G-CSF therapy on monocyte function of 32 patients with refractory testicular cancer [12]. GM-CSF-treated patients exhibited enhanced levels of expression of MHC class I and II antigens, whereas G-CSF treatment enhanced only MHC class I antigen levels. In addition, the allogeneic tumoricidal activity of monocytes against TNF-sensitive U937 leukaemic cells was enhanced by GM-CSF therapy while G-CSF was without effect. Wing and colleagues have shown that in patients with refractory neoplastic disease, GM-CSF treatment increased the secretion of TNF following secondary stimulation with LPS and an increase in monocyte ADCC against antibody-coated xenogeneic cells *in vitro* [13]. Chachoua and colleagues also found that monocyte activation was achieved in 24 patients with solid malignancies treated systemically with GM-CSF [14]. 15 of these patients exhibited an enhanced cytotoxicity against allogeneic human colon carcinoma HT29 cells. Other studies have shown that monocyte-mediated anti-leukaemic killing by cell-to-cell contact was more effective than cell-free secreted cytokines such as TNF [15] and this was concordant with our investigations [4, 7]. We suggest that rhGM-CSF administration may directly facilitate the monocyte tumoricidal activity, particularly during recovery from intensive chemotherapy where the tumour load would be predicted to be minimal.

Table 1. *Monocyte-mediated cytolysis of leukaemic cell lines* in vitro

Treatment group	K562 (% cytolysis)	U937 (% cytolysis)	KG-1 (% cytolysis)
Control	21.6±10.2	42.2±12.1	56.3±12.2
GM-CSF	47.3±10.6*	76.3±14.6*	84.1±17.1*
G-CSF	25.6±10.2	44.9±15.2	60.2±14.9
LPS	44.8±12.9*	74.1±16.2*	81.1±15.6*
GM-CSF/LPS	77.1±14.1†	89.6±10.6†	95.2±10.0†
G-CSF/LPS	51.1±13.9*	76.4±18.4*	85.5±16.9*

The effect of rhGM-CSF or rhG-CSF on the ability of monocytes to kill leukaemic targets *in vitro*. Figures are per cent leukaemic cells killed by activated monocytes by a lactate dehyrogenase release (LDH) assay where membrane-perturbed or dead cells release this otherwise stable cytosolic enzyme. *Denotes significantly different from control ($P<0.01$). †Denotes significantly different from either agent alone ($P<0.01$).

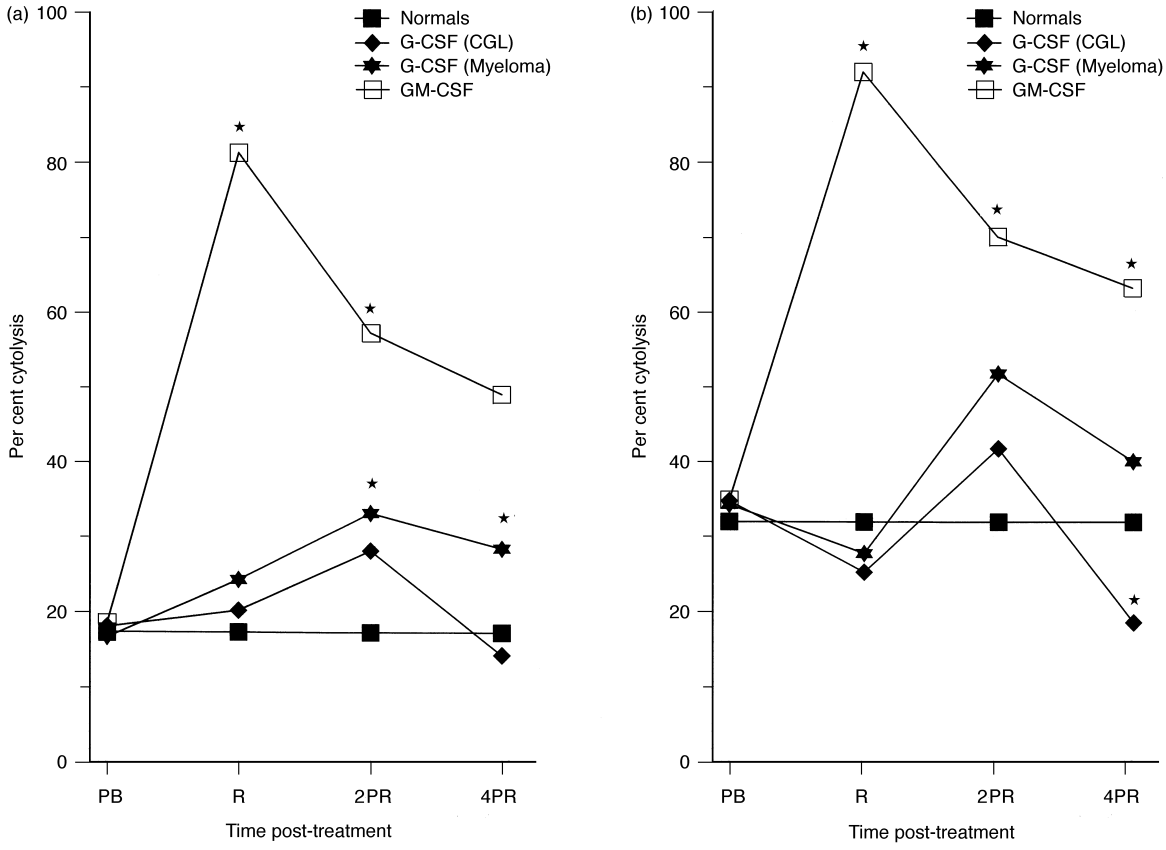

Figure 2. The effect of rhGM-CSF or rhG-CSF administration and monocyte-mediated cytolysis of K562 targets. The LDH cytolysis assay was used as a functional readout of the anti-leukaemic function of monocytes. Monocyte-mediated killing without LPS co-stimulation (a) and with LPS co-stimulation (b) is shown. All patients and normal control subjects exhibited similar killing activities for K562 cells prior to chemotherapy. However, while there was no observed deficit in monocyte-mediated killing from G-CSF-treated patients, those patients treated with rhGM-CSF exhibited an enhanced effect against K562 at all time points at and post-regeneration, as compared with rhG-CSF-treated. patients (*P<0.001). rhGM-CSF primed monocytes respond to secondary stimulation by LPS (b) whereas rhG-CSF was without effect. Error bars have been omitted for clarity.

IMMUNOTHERAPEUTIC STRATEGIES AGAINST CANCER UTILISING THE MONOCYTE SYSTEM

Manipulation of either the tumour or the primary effector cell may offer greater opportunities in the management of human cancer than is currently possible with conventional or high-dose chemotherapy. By inducing an activated immune response against cancer cells that survive high-dose chemotherapy (minimal residual disease) it may be possible to extend disease-free survival in certain patient groups. Direct activation of the monocyte *in vivo* by administration of GM-CSF is possible [1, 4, 7, 12–15]. Faradgi and colleagues have also described a phase I trial of intravenously infused *ex vivo* activated monocytes in the treatment of patients with non-small cell lung carcinoma [16]. Monocytes activated with IFN-γ were administered intravenously to 11 patients once a week for 6 consecutive weeks. This approach was deemed safe and suggested uptake of activated monocytes at the site of the tumour. Immunomodulatory activity of the infused monocytes was confirmed in that increased granulocyte counts and neopterin were found in the blood of patients and enhanced secretion of IL-1, TNF and neopterin were quantitated in culture supernatants of monocytes.

Adoptive transfer of cytotoxic monocytes and macrophages may contribute to an antitumour effect and contribution to the disease-free survival of the patient [17–19]. Boccoli and colleagues adoptively transferred human monocytes into cancer patients and reported activation of monocytes *in vivo* following high-dose interleukin-2 bolus treatment [17]. Chokri and colleagues obtained tumoricidal macrophages from one cytapheresis by *ex vivo* culture and adoptive transfer to a patients with metastatic cancer was not associated with dose-limiting toxicity [18]. The tumoricidal macrophage yield was improved following co-culture with GM–CSF and dihydroxy-cholecalciferol. This also resulted in a higher tumoricidal activity of monocytes against human tumour cell-lines *in vitro* following stimulation with IFN-γ. In addition, a phase I trial of adoptive immunotherapy with *in vitro* IFN-γ and LPS activated monocyte-derived macrophages in 9 patients with solid malignancies demonstrated the feasibility of such an approach which is also tolerated without major side-effects [19]. One patient with previously progressing colorectal cancer exhibited stable disease following this form of therapy [19].

CONCLUSION

Monocytes and their communication with other cells of the immune system play a key role in the regulation of host immunity against infection and malignancy. Selective modulation of their function, particularly by the erogenous therapeutic use of GM-CSF may have applications in those patients undergoing cancer chemotherapy, especially in the context of minimal residual disease. The availability of

recombinant cytokines and technology for gene transfection has led to renewed optimism in the application of immunotherapeutic strategies for a whole spectrum of diseases. This is complemented by a resurgence of clinical programmes involving monocyte activation as well as the manipulation of other immunological systems. Refinements in these protocols and a better understanding of the biological processes involved in the cellular and molecular regulation of host immunity may yield genuine and sustained benefits where current conventional therapies have failed or are unlikely to adequately deal with the underlying progression of the malignant state.

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