Dual Role of GM-CSF as a Pro-Inflammatory and a Regulatory Cytokine: Implications for Immune Therapy

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Granulocyte macrophage colony stimulating factor (GM-CSF) is generally recognized as an inflammatory cytokine. Its inflammatory activity is primarily due its role as a growth and differentiation factor for granulocyte and macrophage populations. In this capacity, among other clinical applications, it has been used to bolster anti-tumor immune responses. GM-CSF-mediated inflammation has also been implicated in certain types of autoimmune diseases, including rheumatoid arthritis and multiple sclerosis. Thus, agents that can block GM-CSF or its receptor have been used as anti-inflammatory therapies. However, a review of literature reveals that in many situations GM-CSF can act as an anti-inflammatory/regulatory cytokine. We and others have shown that GM-CSF can modulate dendritic cell differentiation to render them “tolerogenic,” which, in turn, can increase regulatory T-cell numbers and function. Therefore, the pro-inflammatory and regulatory effects of GM-CSF appear to depend on the dose and the presence of other relevant cytokines in the context of an immune response. A thorough understanding of the various immunomodulatory effects of GM-CSF will facilitate more appropriate use and thus further enhance its clinical utility.

Introduction

GRANULOCYTE MACROPHAGE colony-stimulating factor (GM-CSF) was first characterized by Burgess, Camakaris, and Metcalf as a soluble factor capable of differentiating bone marrow precursor cells into granulocytes and macrophages (Burgess and others 1977). It is now recognized as an immune modulatory cytokine produced by different cells, including macrophages, endothelial cells, alveolar epithelial cells, and T cells (Kelso and Metcalf 1985; Hamilton 2002; Fleetwood and others 2005; Ponomarev and others 2007). GM-CSF production can be regulated by cytokines, antigens, or other inflammatory agents. While inflammatory cytokines such as interleukin (IL)-1, IL-2, tumor necrosis factor- α (TNF α), and interferon- γ (IFN- γ) can induce GM-CSF production and secretion, immune-regulatory cytokines such as IL-10 and transforming growth factor- β (TGF β) can suppress its production (Lari and others 2007; Hamilton 2008; Ogawa and others 2008).

GM-CSF plays a dominant role in the survival, proliferation, differentiation, and function of myeloid lineage cells (Hamilton 2008). Due to its pleiotropic effects on different cell lineages, the therapeutic potential of GM-CSF has been explored in different conditions such as inflammation, cancer, and autoimmunity (Hamilton 2008; Metcalf 2010; Di

Gregoli and Johnson 2012; Zhan and others 2012). Consistent with this, some studies have suggested that GM-CSF causes, or is a part of, an inflammatory response (El-Behi and others 2011; van Nieuwenhuijze and others 2013), while others suggest that GM-CSF promotes immunological tolerance by acting as an immunoregulatory cytokine (Parmiani and others 2007; Kohanbash and others 2013). As has been shown for other cytokines that have both pro-inflammatory and regulatory properties, the effects of GM-CSF are likely dose and context dependent (Parmiani and others 2007; Shachar and Karin 2013). Here, we limit ourselves to providing a review and analyses of the literature that may help better understand how the apparent “duality” of GM-CSF function is brought about.

Nature and Properties of GM-CSF

GM-CSF is a glycoprotein consisting of 127 amino acids with 2 potential N-linked glycosylation sites (Kaushansky and others 1987; Cebon and others 1990). Although the glycosylation sites do not appear to be essential for GM-CSF activity *in vitro* or *in vivo*, they appear to affect GM-CSF's affinity for its receptor. The crystal structure of recombinant human GM-CSF has shown that it consists of 4 anti-parallel helical bundles (Walter and others 1992).

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The GM-CSF receptor (GM-CSFR) consists of 2 distinct chains. The GM-CSF receptor α -chain (GMR α) directly binds GM-CSF, although with lower binding affinity ($K_d=2-8$ nM) while displaying rapid dissociation kinetics (Gearing and others 1989; Park and others 1992). The GM-CSF receptor β -chain alone (GMR β) does not exhibit any measurable binding affinity for GM-CSF. However, on its interaction with GMR α , it converts the pair into a higher-affinity receptor for the GM-CSF ($K_d=30-100$ pM) with slower dissociation kinetics (Cannistra and others 1990; Chiba and others 1990; Hayashida and others 1990; Park and others 1992). However, the GMR α -chain confers GM-CSF-binding specificity to GM-CSFR (Hara and Miyajima 1992; Takaki and others 1994); the GMR β -chain, in addition to its ability to associate with GMR α , can also associate with IL-3 and IL-5-specific receptor subunits and enhance the affinity of those ligand-receptor interactions. For this reason, IL-3, IL-5, and GM-CSF are considered members of the β common (β_c) family of cytokines (Broughton and others 2012; Hercus and others 2013), and their sharing of this receptor subunit may explain the partial cross-reactivity for receptor binding displayed by these cytokines (Walker and others 1985; Lopez and others 1989). GMR α and the β chains are predominantly expressed in various cells of the hematopoietic lineage; however, they have been also found to be expressed in nonhematopoietic cell lines, such as small cell carcinomas, which bind endogenous GM-CSF, leading to the activation of downstream signaling pathways (Dedhar and others 1988; Baldwin and others 1989; Hercus and others 2009; van de Laar and others 2012).

GM-CSFR-mediated signaling appears to be initiated by trans-phosphorylation of Janus Kinase 2 (JAK2), which is associated with the β_c receptor subunit. GM-CSF binding to the GMR α subunit facilitates its interaction with the GMR β subunit, thus activating the GM-CSFR-mediated signaling (Hansen and others 2008; Hercus and others 2013). The activated JAK2 phosphorylates multiple tyrosine residues in the GMR β , triggering the activation of downstream signaling cascade, including JAK2/STAT5, Ras/MAP-kinase, and PI3-kinase/Akt (Hercus and others 2009). While GM-CSF induced PI3K-Akt activation has been shown to protect cells from apoptosis (Guthridge and others 2004), ERK and STAT5 activation have been shown to be required for cell proliferation (Comalada and others 2004) and dendritic cell (DC) differentiation (Sebastian and others 2008), respectively. In addition, GM-CSF has been found to activate NF- κ B directly through an interaction of the GMR β with IKK2 (Ebner and others 2003; Perugini and others 2010) or indirectly through JAK2 activation (Guthridge and others 2004), leading to cell survival and proliferation (Ebner and others 2003).

Primary actions of GM-CSF include the regulation of cell survival, proliferation, and differentiation in granulocyte-macrophage populations (Metcalf 1988; Metcalf and Moore 1988). Withdrawal of GM-CSF from purified hematopoietic progenitor cells *in vitro* or from GM-CSF dependent cell lines leads to loss of cell viability (Metcalf and Merchav 1982). In the presence of GM-CSF, the cells survive and proliferate with extended life spans. GM-CSF-mediated prolongation of the half life of myeloid effector cells such as granulocytes and basophils *in vivo* may be important for augmenting host inflammatory responses (Begley and others

1986; Colotta and others 1992). Interestingly, GM-CSF has been shown to have a dose-dependent effect; at low concentrations, it has been shown to support cell survival without proliferation, while at higher concentrations, GM-CSF promotes both survival and proliferation (Guthridge and others 2006).

In addition to its effects on normal cells, a number of immortalized and growth factor-dependent cell lines proliferate with minimal differentiation in response to GM-CSF (Berdel and others 1990; Brizzi and others 1990). While GM-CSF can cause granulocyte lineage commitment at higher concentrations, it can cause monocyte lineage commitment (Metcalf 1980) at lower concentrations. Therefore, it is suggested that not only GM-CSF induces differentiation of specific myeloid cell types but also selectively guides the differentiation of a common progenitor along the granulocyte, macrophage, and DC development pathways in a concentration-dependent manner.

Role of GM-CSF in Inflammation and Autoimmunity

GM-CSF was first characterized as a pro-inflammatory cytokine due to its ability to stimulate plasminogen-dependent fibrinolysis activity in mouse macrophages (Hamilton and others 1980). Ever since, it has been consistently depicted as a pro-inflammatory cytokine that is important in many cellular processes such as DC activation, granulocyte survival, and enhancement of macrophage and microglial functions (Colotta and others 1992; Fischer and Reichmann 2001; Fleetwood and others 2005; Francisco-Cruz and others 2014). In a typical inflammatory response, DCs and macrophages play critical roles in linking the innate and adaptive immune responses. DCs are among the first cells to capture, process, and present antigens to naïve T cells, while concurrently expressing the co-stimulatory molecules necessary for the activation of T cells and initiation of the inflammatory response. The interaction between the antigen presenting cells (APCs) and T cells is influenced by GM-CSF through upregulation of the major histocompatibility complex class II molecules (MHC class-II), necessary for antigen presentation, and by enhancing the secretion of cytokines, including TNF α , IL-6, and IL-23, (Miller and others 2002; Mausberg and others 2009). Other studies have shown that GM-CSF treatment in conjunction with microbial stimuli can support the generation of potent effector DCs that are capable of secreting a variety of pro-inflammatory cytokines that guide the differentiation of T cells during the immune response into effector T cells (Min and others 2010).

Dysregulation of GM-CSF expression has been implicated in the pathogenesis of autoimmune inflammatory diseases (Cornish and others 2009). Several studies have suggested that GM-CSF can indeed exacerbate autoimmune diseases such as rheumatoid arthritis (RA), a chronic inflammatory disorder characterized by joint pain and deterioration (Fiehn and others 1992; Alsalamah and others 1994; Berenbaum and others 1994; Burmester and others 2013). The chronic pain and gradual destruction of the joints is mediated by macrophages, through the production of a wide range of inflammatory cytokines, such as TNF α , IL-1 β , and IL-6 (Bischof and others 2000; Cook and others 2001). Two distinct macrophage lineages have been described: M1

lineage, which is involved in inflammation and host defense, and M2, which is characterized as anti-inflammatory and involved in tissue repair processes (Mantovani and others 2004). GM-CSF, which is found at higher levels in patients with RA, has been suggested to promote the development of M1 macrophages (Verreck and others 2004). Preclinical studies to establish a connection between GM-CSF and RA found that GM-CSF injections in a mouse model of collagen-induced arthritis (CIA) (Seki and others 1988) exacerbated the disease (Campbell and others 1997; Campbell and others 1998), while treating mice with CIA with neutralizing antibodies against GM-CSF prevented disease progression. The importance of GM-CSF in exacerbating CIA has been further substantiated by the observed resistance of GM-CSF knockout mice to CIA (Campbell and others 1998).

Inflammatory activity of the GM-CSF has also been implicated in the pathogenesis of Experimental Autoimmune Encephalomyelitis (EAE), an autoimmune disorder of the central nervous system (CNS) that serves as a model of Multiple Sclerosis (McQualter and others 2001; Ponomarev and others 2007). While EAE is widely believed to be a T-cell-mediated autoimmune disease (Kroenke and others 2008), T-cell activation is unlikely due to the direct effects of GM-CSF on mouse T cells as they do not generally express the GMR α (Rosas and others 2007). However, recent reports have suggested different mechanisms through which GM-CSF can drive inflammation in the CNS (Codarri and others 2011; El-Behi and others 2011) (Fig. 1).

IL-17 producing Th17 cells have been strongly implicated in the pathogenesis of both EAE and multiple sclerosis (Muls and others 2012; Kang and others 2013). Further, TGF- β and IL-6 have been implicated in the generation of Th17 cells (Bettelli and others 2006). GM-CSF has been implicated in both the regulation of pro-inflammatory activity of TH17 cells and the differentiation of TH17 cells indirectly through the stimulation of IL-6 secretion by APCs (Sonderegger and others 2008; Ko and others 2014). The

cytokine IL-23 has also been shown to be critical in the “terminal differentiation” of TH17 cells (McGeachy and others 2009). Recent studies have shown that IL-23 can induce GM-CSF secretion by Th17 cells, which, in turn, can stimulate the production of IL-23 by APCs (El-Behi and others 2011). GM-CSF-mediated induction of IL-23 as well as IL-6 and other pro-inflammatory cytokines by APCs could result in continued activation as well as in *de novo* generation of Th17 effector cells. Thus, the IL-23/GM-CSF positive-feedback loop could help perpetuate and sustain the inflammatory cycle (El-Behi and others 2011) (Fig. 1A).

An alternative model suggests a different mechanism by which GM-CSF can drive inflammation. GM-CSF has been identified as the cytokine necessary for the recruitment of myeloid cells into the CNS and the development of EAE (Codarri and others 2011). GM-CSF-deficient mice are resistant to developing many experimental autoimmune diseases, including EAE, myocarditis, and CIA (Campbell and others 1998; McQualter and others 2001; Sonderegger and others 2008). The CNS of GM-CSF-deficient mice primed with EAE-specific antigen exhibited lower expression of MHC class II in microglia and had fewer infiltrating macrophages compared with similarly immunized WT mice. Although the expression of the GM-CSF receptor on microglia was not required for EAE induction, the accumulation of microglia was critical and was dependent on GM-CSF-mediated signaling (Fig. 1B). However, the precise mechanism by which GM-CSF promotes the infiltration of macrophages into the CNS is yet to be investigated (Codarri and others 2011).

In the same study, GM-CSF-secreting CD4⁺ T cells were shown to express retinoic acid receptor-related orphan receptor- γ t (ROR γ t), which earlier had been shown to be critical for Th17 cell function (Ivanov and others 2006). Further, while ROR γ t-deficient T cells failed to produce GM-CSF, induction of ROR γ t expression resulted in GM-CSF production. Taken together, Th17-related transcription

FIG. 1. The pro-inflammatory role of granulocyte macrophage colony stimulating factor (GM-CSF) in experimental autoimmune encephalomyelitis. (A) GM-CSF produced by TH17 cells induces antigen presenting cells to secrete interleukin (IL)-23 (and IL-6), which supports the maintenance (and differentiation) of TH17 cells in a feedback mechanism, resulting in inflammation and autoimmunity. (B) GM-CSF secreted by TH17 induces the migration of myeloid cells into the central nervous system (CNS) and causes inflammation and autoimmunity.

factor ROR γ t appears to be involved in the regulation of GM-CSF expression. Collectively, these data support the claim that GM-CSF is an important pro-inflammatory mediator in certain autoimmune disorders.

Role of GM-CSF in Immune Tolerance

In contrast to the role of GM-CSF as a pro-inflammatory cytokine, recent studies have described GM-CSF as a cytokine involved indirectly in the induction of immunological tolerance and anti-inflammatory responses. Unlike IL-10 and TGF β , both of which demonstrate physiological anti-inflammatory and immunosuppressive functions, the GM-CSF has not been considered a tolerogenic or immunosuppressive cytokine (Hillyer and others 2006). However, GM-CSF has been shown to facilitate T-cell-mediated tolerance by inducing "tolerogenic" DCs (Steinman and others 2003). DCs differentiate from a population of precursor cells, and they undergo either terminal differentiation or maturation for efficient antigen presentation. This maturation process is characterized by enhanced expression of MHC class II, B7 family co-stimulatory molecules, and an array of pro-inflammatory cytokines such as IL-12, which enhances the inflammatory response (Inaba and others 1994; Koch and others 1996; Inaba and others 2000). Antigen presentation by matured DCs leads to T-cell activation and pro-inflammatory responses. The critical role of DC development in T-cell activation has been further established by studying the effects of low antigen dose *in vivo* in the absence of the maturation signals. Low levels of antigens without the accompanying maturation signals can result in incomplete maturation of DCs, which fail to activate T cells and result in immune tolerance (Hawiger and others 2001; Gilliet and others 2002). It has been suggested that these maturation-resistant DCs can effectively induce tolerance by failing to deliver the required "strength of signal" to activate T cells. Thus, DCs in this context may be described as "tolerogenic" (Steinman and others 2003).

Tolerogenic DCs can induce peripheral tolerance through a variety of mechanisms. Sub-optimal antigen presentation by tolerogenic DCs can directly cause T-cell hypo-responsiveness and/or anergy (Kriegel and others 2012; Mayer and others 2012; Raich-Regue and others 2012). Alternatively, tolerogenic DCs can expand or induce regulatory T-cells, which, in turn, can suppress the T-effector cells (Bhattacharya and others 2011). Thus, the means by which tolerogenic DCs induce peripheral tolerance is well understood, but how DC precursors develop into tolerogenic DCs is relatively unstudied.

The basic understanding of DC-mediated tolerance stems from the notion that semi-developed/matured DCs are responsible for developing T-cell tolerance (Rutella and others 2006). Various cytokines, including TNF α , granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and GM-CSF, can modulate DC development into a tolerogenic phenotype (Rutella and Lemoli 2004; Rutella and others 2004; Gangi and others 2005; Li and others 2005; Verginis and others 2005). While bone marrow progenitor cells treated with low-dose GM-CSF can develop into tolerogenic immature DCs, cells treated with a higher dose of GM-CSF can develop into mature pro-inflammatory DCs (Lutz and others 2000). These low-dose GM-CSF-derived tolerogenic DCs have

been reported to be maturation resistant when challenged with standard DC maturation agents such as LPS and TNF α (Berger and others 2009; Guindi and others 2012). Furthermore, these cells failed to upregulate the expression of co-stimulatory molecules or increase the secretion of IL-12 on appropriate stimulation. Interestingly, when these "immature" DCs were adoptively transferred, they were able to home to T-cell areas of the lymphoid organs in the recipient mice (Lutz and others 2000). Moreover, these immature DCs were able to induce T-cell hypo-responsiveness *in vitro* and prolong allograft survival *in vivo*, both definitive indications of immune tolerance (Lutz and others 2000).

A related but alternative mechanism of T-cell tolerance that involves "semi-mature DCs" has been proposed (Verginis and others 2005). GM-CSF exposed bone marrow cells have been shown to develop into semi-mature DCs, which are characterized by increased levels of expression of MHC class-II and CD80/86 co-stimulatory molecules, but lower levels of expression/production of inflammatory cytokines such as TNF α , IL-12, IL-1 β , and IL-6 (Verginis and others 2005). These semi-matured DCs pulsed with auto-antigen could cause selective activation of autoantigen-specific CD4⁺CD25⁺ regulatory T-cells that expressed Glucocorticoid-induced TNF-Receptor (GITR), Cytotoxic T-lymphocyte antigen-4 (CTLA-4), and the transcription factor Foxp3. These regulatory T-cells secreted IL-10 and suppressed proliferation of antigen-specific CD4⁺CD25⁻ T-effector cells. In addition, adoptive transfer of antigen pulsed semi-matured DCs prevented the induction of Experimental Autoimmune Thyroiditis (EAT) by increasing autoantigen-specific Tregs (Verginis and others 2005).

Direct administration of "low-dose" GM-CSF into mice can prevent as well as suppress ongoing EAT (Vasu and others 2003). GM-CSF-induced suppression of EAT was associated with a selective expansion of CD4⁺CD25⁺ T regulatory cells that suppressed autoantigen-specific responses through increased production of IL-10 (Gangi and others 2005). Furthermore, treatment with low-dose GM-CSF can reverse experimental autoimmune myasthenia gravis (EAMG) in C57BL/6 mice and prevent the development of type-1 diabetes (T1D) in NOD mice (Gangi and others 2005; Sheng and others 2006; Cheatem and others 2009). These studies revealed that low-dose GM-CSF acted on DC precursors *in vivo* and caused expansion of a subset of CD11C⁺CD8 α ⁻ DCs (Cheatem and others 2009). These CD8 α ⁻ DCs expressed very low to negligible levels of pro-inflammatory cytokines such as TNF α , IL-1 β , and IL-6, but they expressed higher levels of TGF β (Ganesh and others 2009). Adoptive transfer of these "tolerogenic" DCs from GM-CSF treated donor mice to recipient mice followed by immunization with mTg led to an expansion of Foxp3⁺ Tregs in the draining lymph nodes and prevented the development of EAT (Ganesh and others 2009).

Subsequent studies with bone marrow cells showed that GM-CSF could differentiate precursor cells *ex vivo* into Bone Marrow-Derived Dendritic cells (G-BMDCs) that could expand natural Tregs and differentiate adaptive Tregs through a different mechanism (Bhattacharya and others 2011). In the first case, G-BMDCs caused selective proliferation of only CD4⁺Foxp3⁺ Tregs, and not CD4⁺Foxp3⁻ Teff, in co-cultures with total CD4⁺ T-cells. Further, this Treg proliferation occurred even in the presence of G-BMDCs derived from MHC class-II^{-/-} mice and thus was

TCR independent. This represented a unique mechanism of G-BMDC-induced Treg proliferation that did not require canonical antigen presentation/activation. In a search for cell surface molecules expressed in G-BMDCs that could interact with Tregs and cause their proliferation, we identified 2 molecules, namely the TNF-family ligand OX40L and the notch-family ligand jagged-1 (Jag-1), to be critically important (Bhattacharya and others 2011; Gopisetty and others 2013) (Fig. 2). Adoptive transfer of only OX40L⁺ Jag-1⁺ G-BMDCs led to *in vivo* Treg expansion, increased production of IL-4 and IL-10, and suppression of EAT in the recipient mice. The cognate receptor for OX40L is OX40 and it is constitutively expressed on Tregs (Vu and others 2007). Furthermore, we found that the most likely cognate receptor for Jag-1 contributing to Treg expansion was Notch3 (Gopisetty and others 2013), which is preferentially expressed in Tregs (Anastasi and others 2003). Based on these observations, we speculate that OX40 and Notch3-mediated signaling pathways may be co-operatively interacting to aid in TCR-independent Treg proliferation.

In addition to the capacity of G-BMDCs to expand natural Tregs in a TCR-independent manner, G-BMDCs secreted high levels of TGF β , which along with TCR stimulation could convert T-effectors into adaptive Foxp3⁺ Tregs. Supplementation of anti-CD3 stimulated CD4⁺CD25⁻ T-cell cultures with supernatant from G-BMDC cultures, and it resulted in robust conversion of Teff into induced T-regulatory cells (iTregs) (Bhattacharya and others 2011). Thus, G-BMDCs can contribute to peripheral tolerance by both expanding the pool of natural Tregs and inducing antigen-specific iTregs (Bhattacharya and others 2011). Other unpublished data from our laboratory suggest that low-dose GM-CSF directly contributes to the development of OX40L⁺Jag-1⁺ DCs *in vivo*, and thus may be responsible for maintaining physiological Treg homeostasis. This would suggest a nonredundant role for GM-CSF in maintaining immune tolerance.

Divergent Effects of GM-CSF Noted in Preclinical and Clinical Studies

GM-CSF is either being used or targeted in a variety of treatment protocols for a wide range of diseases, including cancer, autoimmune diseases, and sepsis-related immune-suppression (Salgia and others 2003; Meisel and others 2009; Eroglu and others 2011; Peng and others 2012; Rowin and others 2012; Behrens and others 2014; Kaufman and others 2014). Most of these treatments assume a pro-inflammatory function for GM-CSF.

Dual roles of GM-CSF in cancer

Since cytokines play a major role in modulating anti-tumor immune response (Candido and Hagemann 2013), cytokines such as IL-2 (Forni and others 1985; Forni and others 1988) and GM-CSF (Dranoff 2002, 2003) have been used in cancer treatment protocols for eliciting a strong anti-tumor immune response. In one preclinical study, modified B16 melanoma cells engineered to express GM-CSF elicited a more potent anti-tumor response than the nonexpressing cells in C57BL/6 mice, (Dranoff and others 1993). In another study, closer examination of tumor histology showed an association of increased expression of GM-CSF with enhanced infiltration of APCs such as DCs and macrophages into the tumor (Armstrong and others 1996). An increase in numbers of infiltrating APCs in the tumor may be due to GM-CSF's ability to prime target cells to enhance the synthesis of IL-8, a chemoattractant for neutrophils and MIP-1 α , a chemoattractant for monocytes and lymphocyte subpopulations (Roberge and others 1998). Consistent with these observations, when vaccinated with irradiated autologous melanoma cells engineered to secrete GM-CSF, 11 of 16 patients with metastatic melanoma showed extensive tumor destruction (Soiffer and others 1998). Similarly, autologous tumor cells modified by adenoviral vectors to

FIG. 2. GM-CSF induces differentiation of precursor cells into tolerogenic dendritic cells. GM-CSF induces differentiation of bone marrow-derived precursor cells into dendritic cells that co-express OX40L and Jagged-1. On cell-cell interaction with Foxp3⁺ nTregs, these ligands signal through their cognate receptors, OX40 and Notch3, respectively, driving the proliferation of nTregs in the presence of IL-2.

secrete GM-CSF were successfully used to vaccinate patients with nonsmall-cell-lung carcinoma (NSCLC) (Salgia and others 2003). In another clinical study, in advanced-stage NSCLC patients, tumor vaccines secreting higher levels of GM-CSF correlated with a better survival outcome (Nemunaitis and others 2004).

In a different approach, DCs pulsed with a specific tumor antigen in the presence of GM-CSF alone or GM-CSF + IL-4 have been used as cancer vaccines for melanoma, renal cell carcinoma, malignant glioma, and metastatic prostate cancer (Murphy and others 1996; Nestle and others 1998; Holth and others 1999; Thurner and others 1999; Yu and others 2001). In multiple clinical trials, metastatic prostate cancer patients injected with autologous APCs precultured with a fusion protein (Sipuleucel-T; approved by the FDA for the treatment of metastatic prostate cancer) of prostatic acid phosphatase (PAP; a protein expressed by prostate cancer cells), and GM-CSF showed prolonged survival (Higano and others 2009; Kantoff and others 2010). However, similar approaches using tumor-lysate-pulsed autologous DCs generated with GM-CSF/IL-4 resulted in either a partial protection in patients with metastatic renal carcinoma (Kim and others 2007) or lung carcinoma (Chang and others 2005), or as shown in another study failed to elicit an adequate immune response as noted in metastatic melanoma patients (Redman and others 2008). In addition, vaccines using GM-CSF-secreting tumor cells have demonstrated unpredictable outcomes. In a phase III clinical trial for metastatic prostate cancer, vaccination with GM-CSF producing prostate tumor cells had adverse outcomes with regard to patient survival (Lassi and Dawson 2010). Similarly, the use of GM-CSF as an adjuvant for cell-based vaccination in patients with melanoma had negative outcomes on survival (Faries and others 2009). Thus, it appears that the outcome of GM-CSF-based strategies for treating cancer is dose dependent and may be influenced by the overall context of the immune response (Bendandi 2009; Eggermont 2009).

The reasons for these diverse outcomes is not clear. Interestingly, some studies suggest that one of the strategies that tumor cells use for immune evasion is to produce GM-CSF (Tsuchiya and others 1988; Bronte and others 1999). Human prostate cancer cells have been shown to express both GM-CSF and its receptor (Rokhlin and others 1996), and their levels of expression have been found to correlate with the stage of advancement of gliomas (Mueller and others 1999). Interestingly, GM-CSF concentrations were found to be higher in the local glioma environment than in the periphery (Kohanbash and others 2013). This study further suggested that GM-CSF can cause suppression of T-cell function through the expression of arginase in a suppressor myeloid subpopulation (Kohanbash and others 2013). In a tumor-vaccine study using modified tumor cells expressing GM-CSF, serum level of GM-CSF that was above a threshold level induced the development of CD11b⁺Gr-1⁺ myeloid suppressor cells that impaired antigen-specific T-cell responses (Serafini and others 2004; Serafini and others 2006). Other studies have shown that GM-CSF secretion by tumor cells can lead to the development of an inhibitory population of CD11b⁺Gr-1⁺ cells, which can result in functional impairment of CD8⁺ T-cells (Gallina and others 2006). Collectively, these data suggest that high concentrations of GM-CSF may induce a myeloid suppressor population that can negatively regulate immune activation (Fig. 3). Although CD11b⁺Gr-1⁺ cells present an immature phenotype when cultured with GM-CSF alone, they can be matured with a combination of GM-CSF and IL-4. This suggests that GM-CSF can cause divergent outcomes based not only on the dose but also on the presence of other cytokines (Bronte and others 1999). Studies involving patients with melanoma or colon cancer have shown that using a sub-cutaneous injection of GM-CSF at a dose above 100 µg is ineffective in enhancing an anti-tumor response (Parmiani and others 2007). In contrast, studies using lower doses of GM-CSF, (i.e., 40–80 µg) have shown increased T-effector-cell

FIG. 3. Secretion of GM-CSF by tumor cells leads to immune evasion. Tumor cell-secreted GM-CSF differentiates precursor cells into myeloid suppressor cells, which inhibit host immune response through different mechanisms, including secretion of *iNOS* and Arginase.

response as an overall marker of immune system activation (Parmiani and others 2007). Collectively, these studies suggest that GM-CSF could act in an inflammatory or immunosuppressive manner, depending on dose and the overall cytokine milieu.

Use of GM-CSF in inflammation and immunosuppression

Although Crohn's Disease (CD) is characterized as an inflammatory disease affecting the gut, treatment with GM-CSF has been successfully used to cause disease remission (Dieckgraefe and Korzenik 2002). It has recently been proposed that the CD develops as a result of immunodeficiency (Roth and others 2011). GM-CSF is a potent growth and differentiation factor for myeloid cells, which are responsible for anti-microbial activity. Therefore, neutralization of GM-CSF by anti-GM-CSF autoantibodies has been proposed to be associated with disease progression and relapse (Dabritz and others 2013). A phase I-II trial of GM-CSF in pediatric CD had encouraging results (Kelsen and others 2010). Similarly, in another phase I-II trial in Japanese patients with CD, GM-CSF therapy improved disease activity scores (Takazoe and others 2009). However, a systematic analyses of multiple randomized trials revealed that GM-CSF treatment in CD did not yield better outcomes relative to the placebo-treated group (Roth and others 2012).

GM-CSF has also been used in the treatment of sepsis-associated immunosuppression, which is characterized by a tolerogenic cytokine milieu, T-cell anergy and hyporesponsiveness, and reduced monocyte lineage cell activity (Hutchins and others 2014). GM-CSF treatment resulted in a favorable outcome as indicated by the restoration of immune competence with an accompanying increase in both lymphoid and myeloid subpopulations, including CD4⁺ T cells, CD8⁺ T cells, and neutrophils (Meisel and others 2009). Furthermore, patients in the GM-CSF treatment group exhibited enhanced levels of TNF α (Meisel and others 2009). These data support a general pro-inflammatory role for GM-CSF. However, in one study on preterm Small for Gestational Age (SGA) babies, GM-CSF treatment did not improve sepsis-free survival (Marlow and others 2013).

Collectively, these experiences suggest that inflammation or immunosuppression may not merely depend on the presence or absence of GM-CSF but may depend on the overall immune environment in the host.

Use of GM-CSF in treating autoimmune diseases

As previously discussed in this review, GM-CSF has been linked to several autoimmune disorders, primarily acting in a pro-inflammatory manner through M1 macrophages and Th17 effector cells. When exploring putative treatment modalities for autoimmune disorders such as RA, a common approach involves blocking antibodies for GM-CSF (Behrens and others 2014). MOR103, a human monoclonal antibody against GM-CSF, has been shown to have moderately positive benefits in patients with RA (Behrens and others 2014). In a phase Ib/IIa, double-blind, placebo-controlled, dose-escalation trial, Behrens and others found a statistically significant improvement in the moderate and high dosage treatment groups as assessed by the disease activity scores. Supporting evidence for this

approach has come from studies in which monoclonal antibody Mavrilimumab, which blocks GMR α , was found to significantly ameliorate the severity of RA in a phase II, randomized, double-blind, placebo-controlled, and dose-escalation trial (Burmester and others 2011; Burmester and others 2013).

In contrast, our laboratory combined clinical assessment scores and an analysis of the relevant lymphoid populations in a patient with myasthenia gravis (MG) who underwent experimental GM-CSF treatment to show that GM-CSF has a potential role in stimulating peripheral tolerance as the means to ameliorating MG (Rowin and others 2012). Three weeks after cessation of GM-CSF treatment, a marked decline in the clinical score was observed (Rowin and others 2012). Before treatment with GM-CSF, the Tregs from this patient had lower levels of Foxp3 expression, as compared with Tregs from healthy controls. On treatment with GM-CSF, the Foxp3 expression was increased and sustained, and Foxp3⁺CD4⁺CD25⁺ Tregs were capable of limiting the proliferative capacities of CD4⁺ T effector cells (Rowin and others 2012). This is consistent with our observation on the effects of GM-CSF on peripheral blood cells *in vitro* (Thiruppathi and others 2012) and our earlier *in vivo* studies in a mouse model of EAMG (Sheng and others 2006; Meriggioli and others 2008; Sheng and others 2008; Sheng and others 2011). Thus, it is important to consider the immunological status of the patient and progression of the autoimmune disorder in determining the potential clinical utility of GM-CSF.

GM-CSF itself is implicated directly in at least one autoimmune disease. Pulmonary Alveolar Proteinosis, an autoimmune disorder characterized by lipid deposition in the alveoli of the lungs, has been associated with GM-CSF deficiency (Venkateshiah and others 2006). In clinical trials, administration of GM-CSF either subcutaneously (Venkateshiah and others 2006) or via inhalation (Tazawa and others 2014) has shown promising results. Some studies suggest that autoantibodies against GM-CSF may be responsible for disease pathology (Costabel and Guzman 2005). It is believed that GM-CSF-dependent development of alveolar macrophages is defective in patients, leading to a defect in the pulmonary clearance of surfactants (Sakagami and others 2010). Consistent with this notion, improvement in lung health in patients with Pulmonary Alveolar Proteinosis has been shown to correlate with reduced anti-GM-CSF autoantibody levels (Ohashi and others 2012).

Other Cytokines That Exhibit Duality of Function

GM-CSF is not the only cytokine that exhibits divergent effects, as many other cytokines have also been shown to have divergent effects (Shachar and Karin 2013). TGF β is generally characterized as a regulatory cytokine because of its role in the conversion of peripheral T-effector cells into adaptive Tregs through the induction of Foxp3 expression. (Chen and others 2003; Li and others 2007). TGF β -mediated conversion of adaptive Tregs (Mucida and others 2009) is enhanced in a milieu that is rich in retinoic acid. These Tregs play an important role in establishing antigen-specific immunological tolerance. However, it is now known that the functional effects of TGF β are context dependent. In mouse, TGF β synergizes with IL-6 while in human TGF β

synergizes with cytokines such as IL-6, IL-1 β , and IL-21, to induce Th17 cells that have been implicated in both inflammation and autoimmunity (Korn and others 2007; Rubtsov and Rudensky 2007; Manel and others 2008; Volpe and others 2008; Yang and others 2008; Benwell and Lee 2010; Maddur and others 2012). These findings suggest that the functional property of TGF β whether as an “inflammatory” or a “regulatory” cytokine is not intrinsic but dependent on the presence of other factors.

IL-10 is believed to be an anti-inflammatory cytokine and has been shown to suppress the function of immune T cells (Chaudhry and others 2011) and the inflammatory function of macrophages (Bogdan and others 1991). Moreover, the suppressive activity of Foxp3⁺ Tregs is dependent on their capacity to secrete IL-10 (Gangi and others 2005). However, IL-10 has also been shown to enhance the expression of MHC class-II molecules in B cells, thereby enhancing their ability to present antigens (Go and others 1990) and produce autoantibodies (Llorente and others 1995).

The best example of a cytokine with profound duality of function is perhaps IL-2. Originally described as a T-cell growth factor, it has been used in cancer therapy to support the growth of immune T cells (Rosenberg and Lotze 1986). However, in mice, deficiency of either IL-2 or its receptor results in lymphoproliferative phenotype, often causing autoimmune-like symptoms (Sadlack and others 1993; Suzuki and others 1995). It is now believed that although IL-2 contributes, it is not essential for effector T-cell proliferation. In contrast, a nonredundant function of IL-2 is to serve as a survival factor for Foxp3⁺ Tregs (Tang and others 2008). A number of recent preclinical and clinical studies have shown that a low dose of IL-2 can support Treg survival and growth while being unable to support significant T-effector proliferation (Grinberg-Bleyer and others 2010; Koreth and others 2011; Saadoun and others 2011).

Even well-characterized Th1-type cytokines such as IL-1 β with an established role in inflammation and immunity (Dinarello 2011) have been shown to have immunomodulatory effects. IL-1 β has been shown to promote Foxp3⁺ expression in activated T cells in the presence of IL-2 and TGF β (Ganesh and others 2011), thus exhibiting a context-dependent regulatory activity.

Conclusion

In summary, studies have shown that although GM-CSF and many other cytokines are assumed to be drivers of inflammatory responses, depending on the dose, the microenvironment, and the presence of other cytokines, some of them can act as regulatory cytokines. However, the underlying mechanisms that determine the pro-inflammatory versus the regulatory effects of GM-CSF are not fully elucidated. One of the factors that contributes to the differential effects of GM-CSF appears to be the dose of the cytokine, whereby a lower dose of GM-CSF maintains a tolerogenic subpopulation of myeloid cells that are involved in Treg homeostasis. At higher doses, GM-CSF can cause myeloproliferation, leading to a robust immune response (Nemunaitis and others 2004). At still higher doses, and above a critical physiological threshold, GM-CSF differentiates myeloid precursors into an immunosuppressive phenotype (Serafini and others 2004). Alternatively, the GM-CSF ac-

tion may be context dependent in that it is known to differentiate myeloid precursors into inflammatory DCs (Naik and others 2006), similar to those found during *Listeria monocytogenes* infection (Zhan and others 1998; Dominguez and others 2011). It is possible that the population of monocytic precursor cells that are exposed to GM-CSF use adequate antigen load as a necessary stimulus for their development into inflammatory DCs. In contrast, without the necessary environmental stimulus, GM-CSF could enable monocyte precursors to develop into a population of immature DCs that can exacerbate the immunosuppression noted in the tumor microenvironment. Thus, the intrinsic property of GM-CSF is neither “inflammatory” nor “regulatory” and its true function is likely determined by the dose, presence or absence of other relevant cytokines and the overall context of the immune response.

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Author Disclosure Statement

The authors have no competing financial interests.

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