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REVIEW

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important hematopoietic growth factor and immune modulator. GM-CSF also has profound effects on the functional activities of various circulating leukocytes. It is produced by a variety of cell types including T cells, macrophages, endothelial cells and fibroblasts upon receiving immune stimuli. Although GM-CSF is produced locally, it can act in a paracrine fashion to recruit circulating neutrophils, monocytes and lymphocytes to enhance their functions in host defense. Recent intensive investigations are centered on the application of GM-CSF as an immune adjuvant for its ability to increase dendritic cell (DC) maturation and function as well as macrophage activity. It is used clinically to treat neutropenia in cancer patients undergoing chemotherapy, in AIDS patients during therapy, and in patients after bone marrow transplantation. Interestingly, the hematopoietic system of GM-CSF-deficient mice appears to be normal; the most significant changes are in some specific T cell responses. Although molecular cloning of GM-CSF was carried out using cDNA library of T cells and it is well known that the T cells produce GM-CSF after activation, there is a lack of systematic investigation of this cytokine in production by T cells and its effect on T cell function. In this article, we will focus mainly on the immunobiology of GM-CSF in T cells.

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Identification of GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was first identified in mouse lung tissue-conditioned medium following lipopolysaccharide injection into mice by its ability to stimulate proliferation of mouse bone marrow cells *in vitro* and generate colonies of both granulocytes and macrophages [1]. Much has been learned about the hematopoietic promoting effect of this heavily glycosylated cytokine. GM-CSF stimulates multipotent progenitor cells depending on its concentration, the proliferation of macrophage progenitors at the lowest doses, followed by granulocyte, erythroid, eosinophil, megakaryocyte and multipotent progenitors [2]. It also stimulates the differentiation of myeloid leukemic cells [3] and controls eosinophil function in some instances.

The molecular cloning of mouse and human GM-CSF in 1985 was achieved using T cell cDNA libraries [4, 5], which immediately permitted large-scale production of recombinant GM-CSF and extensive *in vitro* and *in vivo* studies of the biological activity of this cytokine. GM-CSF is encoded by a 2.5 kb mRNA comprising 4 exons. It is secreted as a monomeric 23 kDa glycosylated small protein. Mature murine GM-CSF has 124 residues and human has 127 residues; both are derived from a precursor containing a signal peptide [6, 7]. Murine and human GM-CSF share modest structural homology at the level of the nucleotide (70%)

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Abbreviations: GM-CSF (Granulocyte-macrophage colony-stimulating factor); APC (antigen presenting cell); TNF (tumor necrosis factor); IFN (interferon); Th1(type I T helper cell); Th2 (type II T helper cell); DC (dendritic cell); TLR (Toll-like receptor); JAK (Janus kinase); MAPK (mitogen-activated protein kinase); STAT (signal transducer and activator of transcription); NK (natural killer cells); CTLA4 (cytotoxic T-lymphocyte-associated protein 4).

and amino acid (56%) sequences. There is no cross-species receptor binding or biological activity, however. GM-CSF is produced by various cell types including macrophages, mast cells, T cells, fibroblasts and endothelial cells [8, 9], mostly in response to immune activation and cytokines that mediate inflammation. It is present in serum and most tissues, and is also found associated with the extracellular matrix and as an integral membrane protein [10].

The biological activities of GM-CSF are exerted through binding to heteromeric cell-surface receptors that are expressed on monocytes, macrophages, granulocytes, lymphocytes, endothelial cells and alveolar epithelial cells [11]. The GM-CSF receptor (GM-CSFR) is composed of a (CDw116; GM-CSFRα) and β (GM-CSFRβc) chains. The βc chain is common to receptors for GM-CSF, IL-3, and IL-5 [12]. Receptor expression is characterized by low number (20-200/cell) and high affinity (Kd = 20-100 pM) [13, 14]. Interestingly, both the α and β chains lack a tyrosine kinase catalytic domain. It has been demonstrated that the βc chain constitutively associates with JAK2. The binding of GM-CSF initiates JAK2 autophosphorylation and post receptor signaling. JAK2 then activates STAT5, and MAPK [15, 16]. Non-JAK2 pathways such as the fps/fes pathway have also been implicated in GM-CSF receptor signaling [17, 18].

Regulation of GM-CSF expression

GM-CSF can be produced by a wide variety of tissue types, including fibroblasts, endothelial cells, T cells, macrophages, mesothelial cells, epithelial cells and many types of tumor cells [7]. In these cells, bacterial endotoxins and inflammatory cytokines, such as IL-1, IL-6, and TNFα, are potent inducers of GM-CSF [19-23]. The regulation of gene expression is exerted at both transcriptional and posttranscriptional levels [7, 24-27]. The IL-3 and GM-CSF genes are closely linked in the genome and reside within a cluster of cytokine genes [28]. Highly inducible expression of both genes at the transcriptional level has been reported. NFAT appears to play a major role in the regulation of GM-CSF expression by the formation of DNase I hypersensitive sites within enhancers [29]. The promoter region of the GM-CSF gene contains a variety of positive and negative regulatory regions [30]. In addition, it is well established that GM-CSF can be controlled by post-transcriptional mechanisms through the AU-rich element (ARE) in its 3' non-coding region [31-34]. Its expression can be inhibited by IL-10 [35], IFNg [36], and IL-4 [37, 38]. In addition, pharmacological agents such as cyclosporine A [29, 39, 40] and glucocorticoids [41-44] are strong inhibitors of GM-CSF expression. Under normal conditions, GM-CSF in the circulation is at low or even undetectable levels, which can rise to high levels in response to immune stimuli such as lipopolysaccharide. It should be noted that polymorphonuclear cells can quickly clear GM-CSF [45]. However, a significant increase of GM-CSF can be found in local tissues, such as the skin of allergic patients with cutaneous reactions and in the asthmatic lung. Arthritic synovial fluid has also been shown to contain measurable GM-CSF, which is expected to contribute to joint and bone destruction. GM-CSF is produced by T cells after TCR activation along with the appropriate co-stimulatory signals. However, the regulation of GM-CSF expression in T cells is not fully understood. High levels are associated with juvenile chronic myeloid leukemia, acute myeloid leukemia, human T leukemia virus infection [46-48] and human immunodeficiency virus infections [49].

Overexpression of GM-CSF leads to severe inflammation

In the past 20 years, studies by different approaches have clearly demonstrated that whenever GM-CSF is overexpressed, pathological changes always follow [50]. Early studies using mice transgenic for GM-CSF showed that overexpression leads to macrophage accumulation, blindness, and severe damages to various tissues [51]. Many cytokines and inflammatory mediators were found to be increased in these mice. GM-CSF overexpression in the stomach leads to autoimmune gastritis [52, 53]. When bone marrow cells infected with a retrovirus expressing GM-CSF were transplanted, a lethal myeloproliferative syndrome was induced [54]. Adenoviral-mediated GM-CSF gene transfer in the lung also led to severe lung eosinophilia, macrophage expansion and fibrotic reactions [55-57]. This information has led to the hypothesis that GM-CSF may have a central role in promoting sensitization to aeroallergens in polluted air [58, 59]. Interestingly, it has been suggested that human GM-CSF polymorphisms are likely asthma determinants [60]. Patients with rheumatoid arthritis who are treated with GM-CSF to correct the neutropenia following cancer chemotherapy can suffer worsened rheumatoid disease [61].

Phenotypes of GM-CSF deficient mice

Mice with homozygous deletion of the GM-CSF gene develop normally and show no significant alterations of hematopoiesis up to 12 weeks of age [62]. Although most GM-CSF-deficient mice are apparently healthy and fertile, all of them surprisingly develop lung abnormality [62]. This coincides with the fact that the very first GM-CSF protein was purified from mouse lung-conditioned medium [1], although very little was investigated about the role of

GM-CSF in the lung biology until recently. In the lung of GM-CSF-deficient mice, there is extensive peribronchovascular infiltration of lymphocytes, predominantly B cells. There are numerous large intra-alveolar phagocytic macrophages in the lung. Some mice show lung infections and inflammation involving bacteria or fungi. Some of these features resemble the pathogenesis of human alveolar proteinosis. Therefore, GM-CSF is dispensable for the maintenance of normal levels of the major types of hematopoietic cells and their precursors in blood, marrow, and spleen. Nevertheless, GM-CSF seems to be essential for normal pulmonary physiology and resistance to local infection. When T cells from these mice were examined for their response to antigenic stimulation, it was found that both Th1 and Th2 responses were diminished [63]. In fact, administration of anti-GM-CSF antibody was found to reduce the protective immune response to *Histoplasma capsulatum*, indicating a critical role of GM-CSF in host defense [64]. Therefore, GM-CSF is critical in the regulation of T cell immune responses.

GM-CSF and T cells

Immediately after its identification, GM-CSF was suggested to be a proinflammatory cytokine [65]. GM-CSF may play a pivotal role in various human inflammatory diseases including rheumatoid arthritis, inflammatory renal disease and inflammatory lung disorders. In a collagen-induced arthritis model, it has been reported that GM-CSF$^{-/-}$ mice develop no disease, and the humoral response to collagen was uncompromised [66]. Interestingly, anti-GM-CSF was found to be more effective than anti-TNF in treating rheumatoid arthritis [67]. Antibody blockade also revealed that GM-CSF is an important mediator in lung inflammatory models and controls neutrophil and macrophage numbers, as well as TLR-4 (Toll-like receptor 4) expression. More dramatically, intranasal administration of anti-GM-CSF abolished airway hyperresponsiveness and airway inflammation caused by diesel exhaust particulates [68]. Similar effects were shown in a murine asthma model [69]. Since T cells play a critical role in the pathogenesis of these immune disorders, these observations clearly indicate a role of GM-CSF in the regulation of T cell function.

Both human and mouse GM-CSF were cloned using cDNA from activated T cells [4, 5]. It is now generally accepted that resting T cells do not express GM-CSF. While various types of T cells produce GM-CSF upon activation, it is mostly a transient event. During viral infection, both $CD4^+$ and $CD8^+$ T cells are known to produce GM-CSF. Interestingly, $CD4^+$ helper T cells of both the Th1 and Th2 type have been shown to secrete GM-CSF. In recent studies, however, GM-CSF has been found to be critical in the regulation of allergic lung inflammation. Several investigators have shown that T cells readily secrete GM-CSF (200-7000 pg/mL) upon stimulation with anti-CD3 [70-72]; its direct effect on T cells remains elusive, however. Since the β chain of the GM-CSF receptor is not expressed by most resting T cells, it is likely that higher than normal levels of GM-CSF are required to trigger the solitary α chain on these cells. This would be consistent with the notion that T cells must be refractory to low levels of GM-CSF in order to avoid over-reaction to the low levels of GM-CSF produced by the innate immune system.

Most of the demonstrated effects of GM-CSF on T cells are believed to be exerted indirectly through antigen-presenting cells (APCs) [63]. It has been shown that GM-CSF is critical for DC development and maturation. In fact, in the protocols for *in vitro* differentiation of DC, GM-CSF is absolutely required. The most studied effects of GM-CSF in the immune system *in vivo* are in anti-tumor immunity, where the interaction between T cells and the APCs is critical. The outcome of this interaction determines the fate of cancer cells depending on whether a "danger" or a "tolerogenic" signal is received by the DCs. The best anti-tumor effect of GM-CSF is achieved when combined with anti-CTLA4. Interestingly, a recent report showed that GM-CSF converted an autoimmune response into an anti-tumor response by increasing DC density in the draining lymphnode, and increasing the frequency of antigen-specific T cells and the amount of IFN-g secretion [73]. Surprisingly, GM-CSF treatment was shown to increase the frequency of $CD4^+CD25^+$ T cells with regulatory properties [74], which is related to the high density of MHC class II and B7 molecules on DCs.

GM-CSF not only has the capacity to increase antigen-induced immune responses, but can also alter the Th1/Th2 cytokine balance. It has recently been shown that mice lacking GM-CSF die rapidly from severe necrosis when exposed to an aerosol delivered infection of *Mycobacteriuin tuberculosis* because of their inability to mount a Th1 response [75]. GM-CSF over-expression, however, failed to focus T cells and macrophages into sites of infection, suggesting that uncontrolled expression of GM-CSF leads to defects in cytokine and chemokine regulation. Therefore, excess GM-CSF does not induce an overly Th1 response and very fine control of GM-CSF is needed to fight infections.

In another study, Stampfli *et al.* [76] demonstrated that adenoviral-based gene transfer of GM-CSF expression promoted a transient increase in IL-4 and IL-5 and an eosinophilic inflammatory response in the lung. They further showed upregulation of DCs and macrophages, as well as the recruitment of $CD4^+$ and $CD8^+$ T cells in the lung. In addition, the CD69 glycoprotein was upregulated, indica-

tive of T cell activation. Alternatively, Barouch *et al.* [77] studied the effect of an augmented $CD4^+$ T cell response elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF, reporting greater than 7-fold increases in IFN-γ levels. These results strongly suggest that GM-CSF can stimulate both Th1 and Th2 type responses depending on the conditions. Furthermore, these studies prove that GM-CSF can alter T cell responses directly or indirectly, as well as providing a link between innate and adaptive immunity. Ahlers *et al*, have shown that complete protection against a recombinant vaccinia virus expressing gp160 can be achieved by the triple combination of GM-CSF, IL-12 and TNF-α, and that GM-CSF enhanced antigen presentation in this study.

In an interesting contrast, a study combining GM-CSF with a DNA vaccine elicited protection against herpes simplex virus infection in the presence of both Th1 and Th2 components. Coinjection of GM-CSF with antigen induced both IL-2 and IFN-γ, and inhibited IL-4 production. This result appears to suggest that GM-CSF promotes Th1 responses, but IgG isotyping showed otherwise as the antibodies were Th2-biased [78, 79]. Thus, GM-CSF seems to favor neither Th1 nor Th2 responses exclusively. Others have also reported either Th1 or Th2-biased responses induced by GM-CSF.

Several models of Th1 and Th2 cell differentiation have been proposed, but the molecular mechanisms controlling this process are still unclear. In addition, the exact mechanisms by which cytokines promote differentiation are still debated, and reports of helper T cell differentiation in the absence of signature cytokines add to the confusion. The presence of a "cytokine-adjuvant" such as GM-CSF raises the possibility that helper T cell differentiation or an entire immune response can bypass the need for the driving cytokines. There is little information about what happens to a majority of T cells that are presented with antigen but without the proper cytokine environment to drive differentiation toward classical Th1 or Th2 type cells. Therefore, it would be important to determine whether activated naïve T cells have a positive feedback mechanism utilizing GM-CSF produced by the T cells themselves. Although activated T cells are one of the major sources of GM-CSF, the specific phenotype of T cells that produce GM-CSF has not been identified. T cells have been shown to lose their ability to synthesize GM-CSF during differentiation. Both Th1 and Th2 cytokines, such as IFN-γ, IL-12, IL-4 and IL-10, negatively regulate GM-CSF production, but the function of GM-CSF is not well delineated in activated or differentiated helper T cells. In addition, the physiological relevance of this cytokine especially in helper T cell responses and homeostasis is largely unknown. A diagram depicting the relationship between GM-CSF and T cells is presented in Figure 1.

Tumor vaccine

Early studies showed that GM-CSF acts as an immune adjuvant to drive both humoral and cellular immune responses. GM-CSF has been reported to initiate the proliferation, differentiation and activation of macrophages, neutrophils, various antigen presentation cells, and to some degree, T cells, in addition to several direct immune-stimulatory functions. Clearly, these properties make GM-CSF a potent adjuvant. In fact, injection of irradiated, GM-CSF transfected tumor cells stimulated an intense local inflammatory reaction consisting of DCs, macrophages, and granulocytes [80, 81]. The activation and accumulation of such large numbers of APC indicates that GM-CSF functioned to increase tumor antigen presentation. Unlike the type of DC accumulation induced by Flt3-ligand overexpression [82], GM-CSF resulted in much higher levels of protective immunity. It seems that GM-CSF may induce a subset of DCs that are superior for the phagocytosis of particulate material, such as dead tumor cells, and that express more costimulatory molecules. An increase in CD1 expression may also lead to greater activation of NKT cells, which play a crucial role in tumor immunity. Interestingly, tumor cells overexpressing GM-CSF could not induce such a high level of anti-tumor immunity in CD1d-deficient mice [83, 84]. In addition to their ability to mobilize and activate DCs and NKT cells, GM-CSF-secreting tumor cells also cause increased production of cytokines such as IL-12 that are required for the activation of $CD4^+$ T cells, which in turn

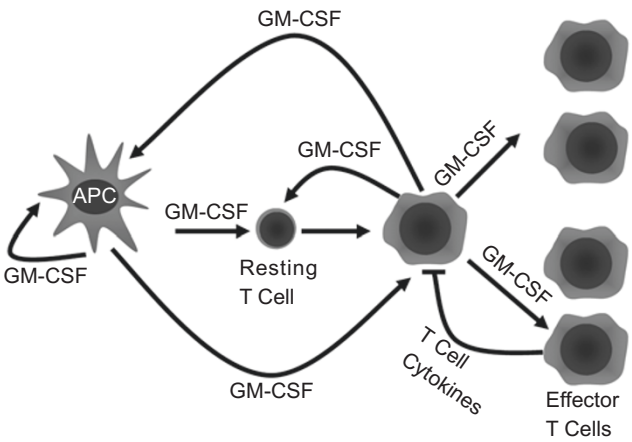

Figure 1 A diagrammatic representation of the role of GM-CSF in T cell responses. APC produced GM-CSF is critical for the activation of resting T cells and the maintenance of APC functions. Specific subpopulations of activated T cells produce significance amount of GM-CSF, which is critical for the function of T cells. In addition, the T cell derived GM-CSF can further enhance the function of APC.

promote cellular immunity and antibody production [85]. Other promising approaches include immunization with antigen fused to GM-CSF [86].

Questions about GM-CSF and T cells

There are many examples of the importance of GM-CSF in inflammatory, infectious and autoimmune diseases. Clearly, GM-CSF can affect various cell types and can promote the survival, proliferation, activation and differentiation of various hematopoietic cell lineages, especially macrophages and DCs. Yet, GM-CSF gene-deficiency does not have dramatic effects on steady state numbers of DCs, raising doubts about a distinct role for GM-CSF in DC development and function *in vivo*. It is well known that many cell types such as lymphocytes, macrophages, fibroblasts, endothelial cells, chondrocytes and smooth muscle cells can make GM-CSF following appropriate stimulation. Very little is known, however, about how GM-CSF production is regulated in the most important producers, the T lymphocytes.

With regard to the link between GM-CSF and T cells, it is odd that we know so little about this important cytokine in this critical population of immune cells. We do not have a clear picture of the conditions and factors that regulate the expression of GM-CSF receptors on T cells. Although activation of $CD4^+$ T cells, $CD8^+$ T cells, and NKT cells leads to the production of GM-CSF, the detailed mechanisms that regulate the expression of this cytokine are poorly understood. While IFN-γ and IL-4 have been shown to inhibit GM-CSF expression, it is unknown whether they account for the transient expression of this cytokine in some T cell populations. Is there a T cell population that is more refractory to downregulation of GM-CSF production by IFN-γ and IL-4? Does GM-CSF produced by T cells play a role regulating the differentiation and function of antigen-presenting cells? Most importantly, can T cells respond directly to GM-CSF? If so, is there a subset difference? Answers to these questions will not only advance our understanding of the basic biology of GM-CSF, but also allow for better clinical applications of this important heterotropic cytokine.

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