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Review

Anti-inflammatory and nitric oxide-inhibiting properties of granulocyte colony-stimulating factor[1]

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KEY WORDS granulocyte colony-stimulating factor; cytokines; infection; inflammation; sepsis; nitric-oxide synthase

ABSTRACT

A proposed scheme between the possible interactions of pro- and anti-inflammatory cytokines, NO and G-CSF during severe inflammation/infection is presented. Taken together, these data indicate that G-CSF exhibits anti-inflammatory properties which may prove to be beneficial in situations associated with an increased activity of the cellular immune system. Since the suppressive effects of G-CSF on the production of pro-inflammatory mediators like TNF-α and nitric oxide are most likely neither cell type nor tissue specific, it is conceivable that NO release induced by pro-inflammatory mediators can be reduced by G-CSF in various organ systems and in different forms of shock. In this context, G-CSF might represent a counterregulatory mechanism directed against a downstream oriented inflammatory response to infection. Therefore, the investigation of G-CSF in the prophylaxis of nonneutropenic infections, sepsis, and other severe inflammatory disorders seems reasonable.

INTRODUCTION

Since the discovery of the granulocyte colony-stimulating factor (G-CSF) some 30 years ago, a plethory of special functions concerning its activity as a haematopoietic growth factor for cells of the neutrophilic lineage have been reported. Therefore, recombinant human G-CSF provided beneficial effects in patients with neutropenia secondary to anticancer chemotherapy, bone marrow transplantations or congenital neutropenia. However, recent studies indicate that, in addition, G-CSF exhibits anti-inflammatory properties which might be therapeutically useful in the prophylaxis of non-neutropenic infections, sepsis, and other severe inflammatory disorders. In this review, we will describe the potential mechanisms of these anti-inflammatory effects of granulocyte colony-stimulating factor. This will include the clinical and experimental data dealing with the influence of G-CSF on pro- and anti-inflammatory cytokines as well as its inhibiting effects on excessive nitric oxide production in different organs and cell lines.

More than 30 years ago Bradley and Metcalf[1] were the first to introduce the term "colony-stimulating factor (CSF)". They could demonstrate a broad variety of CSF producing cells and the occurrence of these factors in several body fluids. A crucial finding for the importance and understanding of CSF in inflammatory/infectious diseases was that endotoxin strongly stimulated the release of CSF. Thus, soon it was suggested that these CSF's were important for the neutrophil response to infectious diseases. As a consequence of the isolation, purification, and cloning of CSF's we now have a class of therapeutic agents with a broad spectrum of application. In this review we

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describe at first biochemical characteristics and signal transduction pathways of granulocyte colony-stimulating factor (G-CSF) and then we focus on recent findings of its anti-inflammatory properties and inhibiting effects on nitric oxide formation.

Biochemical characterization of G-CSF

The human G-CSF gene is localized on chromosome 17, the respective mouse gene is situated on chromosome 11[2,3]. Both consist of ~2.5 kb and five exons[4,5]. Transcriptional activation of the gene is initiated by a number of agonists in various cell types. In monocytes and macrophages, the predominant inducers of G-CSF gene expression are bacterial lipopolysaccharide (LPS) as well as proinflammatory cytokines like *interleukin-1β* (*IL-1β*), *tumor necrosis* factor-α (TNF-α), and interferon-γ (IFN-γ)[6-9]. Although monocytes and macrophages are regarded to be the major source of G-CSF, other cells have been reported to produce and release considerable amounts of G-CSF following LPS- and/or cytokine-stimulation, eg endothelial cells[10], fibroblasts[11], mesothelial cells[12], T-lymphocytes[13], and bone marrow stromal cells[14]. A sequence region located approximately 0.3 kb 5′ of the transcription start site of the G-CSF gene has been identified as obligatory for transcriptional induction of G-CSF. Among others, this region contains cis-elements for the binding of nuclear factor kappa B (NF-κB), NF-IL-6/C-EBPβ, and octamer transcription factor[11]. In addition, a constitutive activity of G-CSF synthesis was described for some tumor cell lines, eg, bladder carcinoma, squamous carcinoma, glioblastoma, and hepatoma[15-16].

Native human G-CSF is an acidic protein consisting of 174 amino acids with a molecular weight of 18.6 kDa. Native murine G-CSF consists of 178 amino acids sharing a 70 % homology with the human protein at the amino acid level. Human G-CSF is *O*-glycosylated at Thr-133, which increases the apparent molecular weight to approximately 19.0 kDa[17]. *O*-glycosylation stabilizes the molecule by suppressing the formation of aggregates [18] and increasing the resistance to protease degradation[19], but is not a prerequisite for regular protein function. This might explain the fact that non-glycosylated pharmaceutical forms of recombinant human G-CSF (eg, filgrastim) share the same pharmacodynamic properties as their glycosylated counterparts (eg, lenograstim).

G-CSF-Receptor and signal transduction

The human G-CSF-receptor gene is situated on chromosome 1, whereas the mouse G-CSF-receptor gene is located on chromosome 4[20,21]. The cDNA of human and mouse G-CSF-receptor show more than 60 % homology[22], which in addition to the above-mentioned similarity of the human and murine G-CSF-protein explains the significant species cross-reactivity [23]. G-CSF-receptor is predominantly expressed on granulocytes and their progenitor cells where receptor number increases with neutrophilic differentiation. Mature human neutrophils display between 200 *and 1000 copies of G-CSF-receptors*[24]. *An increase* in G-CSF-receptor count was observed in promyelocytic leukemia cell lines treated with retinoic acid[25], whereas agents like LPS, TNF-α, granulocyte-macrophage-CSF (GM-CSF), complement factor C5a, and phorbol esters have been reported to downregulate G-CSF-receptor expression on neutrophils[26]. Apart from the neutrophilic lineage, G-CSF-receptors were found on various other cell types including monocytes, macrophages, platelets, endothelial cells, and small lung cell carcinomas[27-29].

The G-CSF-receptor belongs to the cytokine receptor superfamily without intrinsic tyrosine kinase activity and is a member of a subgroup of single-chain receptors as are receptors for erythropoietin, thrombopoietin, growth factor, and prolactin[30]. Due to different mRNA splicing, five different human G-CSF-receptor isoforms have been identified to date[31]. One of them encodes a soluble receptor, which may probably function as a binding protein that protects G-CSF from degradation. The four membrane-bound receptor classes are supposed to differentially regulate G-CSF signal transduction in the respective cell lines. The precise signal transduction pathways modulated by G-CSF are only rarely understood. Studies with different G-CSF-receptor deletion mutants have revealed the presence of distinct functional regions within the cytoplasmic receptor domain responsible for the transduction of proliferative or maturation signals[32-33].

Different models for signalling cascades through

the G-CSF-receptor include tyrosine phopsphorylation and activation of tyrosine kinases, especially JAK2[34] which may be a first step in activation of STAT-3 observed in $CD34^+$-progenitor cells in response to G-CSF treatment[45]. In addition, activation of $p21^{ras}$ and the MAP kinases p42, p44 in response to G-CSF was described[43,46]. Although the precise roles of these pathways in G-CSF signal transduction are not clearly understood, the presence of discrete functional domains in the cytoplasmic region of the G-CSF-receptor indicates that coupling of G-CSF to its receptor may activate various of these signalling pathways. This may at least in part explain the sometimes contradictory effects of G-CSF on different cell types described in the following sections.

Anti-inflammatory mechanisms of G-CSF

In the following sections, the potential anti-inflammatory and beneficial properties of G-CSF based on clinical and experimental data will be presented. Due to these effects, clinical applications of G-CSF even in non-neutropenic infections, especially in patients with septic shock, seems reasonable. However, the exact mechanism (s) of the salutary effects of G-CSF are at present unclear. In general, anti-infectious actions might be mediated by the downregulation of pro-inflammatory agonists and/or the upregulation of their antagonists.

Animal and cell culture experiments Concerning animal studies, *ex vivo* LPS stimulated TNF-α release from rodent macrophages, which were prepared from donor animals and pretreated with G-CSF, was found to be significantly suppressed as compared to cells from control animals[37]. In a rat model of splanchnic artery occlusion shock, application of G-CSF reduced serum TNF-α levels[38]. Improved survival rates, decreased count of bacterial colony forming units, lower serum levels of TNF-α, and higher serum levels of the anti-inflammatory cytokine IL-10 were reported in a murine model of polymicrobial peritonitis and sepsis following a combined treatment of the animals with G-CSF and antibiotics[39]. The most dominant anti-infectious effects of G-CSF could be obtained when the treatment started before the onset of peritonitis and sepsis induced by cecal ligation and puncture. Thus, a prophylactic regimen with G-CSF in addition to antibiotics might represent an improvement in the management of infections that follow abdominal surgery. Comparable results were reported by Lorenz *et al*[40], who found decreased mortality rates in rats pretreated with G-CSF before intraperitoneal feces challenge. This was accompanied by a diminished serum TNF-α-concentration. Furthermore, prophylactic G-CSF administration reduced endotoxemia and serum TNF-α-levels and improved cardiac function and survival rates in a canine model of bacterial pneumonia induced via intrabronchial inoculation with *E coli*[41]. However, similar improvements in cardiovascular functions and endotoxin clearance, but no alteration of serum TNF-α levels were observed in canines challenged with intravenous endotoxin following G-CSF treatment. These data suggest that the beneficial effects of G-CSF are not strictly dependent on lowering TNF-α production and/or release.

In our laboratories, *in vitro* studies dealing with the potential anti-inflammatory properties of G-CSF have been performed with the human hepatoma cell line HepG2. In these experiments, IL-1-induced TNF-α gene expression and protein synthesis by HepG2 cells was significantly suppressed by coincubation with rhG-CSF[42] (Fig 1).

rhG-CSF in healthy volunteers Interactions between recombinant human G-CSF (rhG-CSF) and pro- as well as anti-inflammatory agonists have been reported by Hartung *et al*[43], who treated healthy volunteers with rhG-CSF prior to *ex vivo* blood incubation with bacterial lipopolysaccharide. They found that rhG-CSF altered cytokine release capacity of whole blood as compared to the placebo controlled subjects. In detail, reduced levels of TNF-α, IFN-γ, and GM-CSF were reported, whereas IL-1 receptor antagonist (IL-1ra), soluble TNF-receptors p55 and p75, interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-10 (IL-10) were augmented. The key finding of this study was that rhG-CSF switched whole blood cells towards an increased synthesis of anti-inflammatory and a decreased release of pro-inflammatory mediators. Pajkrt *et al*[44] detected different properties of rhG-CSF in human volunteers inoculated with *E coli* LPS when administered intravenously 2 h and subcutaneously 24 h prior to endotoxin challenge. Applicated 2 h before LPS,

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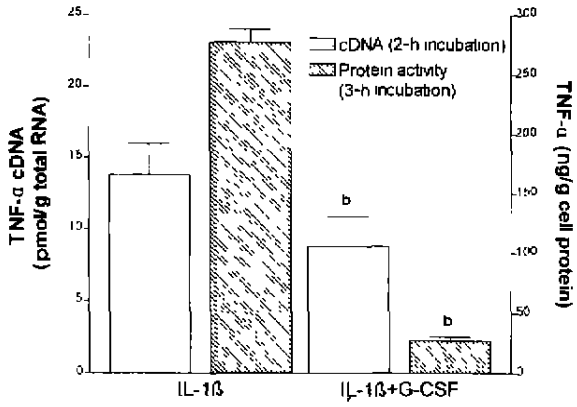

Fig 1. Inhibitory effect of granulocyte colony-stimulating factor (G-CSF) on tumor necrosis factor-α (TNF-α) gene expression (left Y-axis) as well as tumor necrosis factor-α protein synthesis (right Y-axis) in the human hepatoma cell line HepG2. Cells were incubated with 10 kU·L^{-1} interleukin-1β (IL-1β) and 10 kU·L^{-1} interleukin-1β + 250 kU·L^{-1} granulocyte colony-stimulating factor (IL-1β + G-CSF) for 2 h (gene expression studies) and 3 h (protein synthesis studies), respectively. Data are given as means ± SD, $n = 5$. $^bP < 0.05$ as compared to the single incubations with interleukin-1β.

rhG-CSF induced an increased release of TNF-α, IL-6, IL-8, IL-1ra, and soluble TNF-receptors p55 and p75. When administered 24 h before LPS, rhG-CSF attenuated systemic TNF-α levels and increased the serum concentrations of IL-1ra as well as soluble TNF-receptors. The authors concluded that rhG-CSF given 2 h before LPS augmented endotoxin-induced inflammatory cytokine responses while an administration 24 h before LPS challenge resulted in an increased anti-inflammatory response.

Clinical evidence for anti-inflammatory properties of rhG-CSF Beneficial effects of rhG-CSF in the neutropenic patient with infectious diseases are well known for several years[45] (for review see Welte *et al*). Recent data indicate that this benefit cannot solely be attributed to the G-CSF induced granulopoiesis with its consequences on neutrophil function. G-CSF alters the inflammatory host response by a more complex fashion than simply boosting the number of primed circulating neutrophils. In neutropenic sepsis patients administration of rhG-CSF attenuated the inflammatory response as indicated by a drop in C-reactive protein, IL-6 as well as IL-8[46]. Similar results were described by Weiss *et al*[47] in non-neutropenic surgical intensive care patients. Beside an increase in leukocyte count, an upregulation in CD64, CD32 and the intercellular adhesion molecule ICAM-1, and a downregulation in LAM-1, IL-8 decreased and IL-1ra increased after prophylactic rhG-CSF application. In addition, the incidence of severe sepsis was lower in the rhG-CSF group. Recently, the same authors[48] detected no apparent effects of rhG-CSF on the serum levels of TNF-α and TNF-receptor p55 in patients at risk of or with sepsis. However, they found an augmentation of IL-1ra following rhG-CSF-treatment which may indicate an amelioration of the deleterious effects of TNF-α and IL-1β. In a recent study the frequency of nosocomial bacteriemias was reported to be diminished after prophylactic application of rhG-CSF in patients with traumatic brain injury[49]. In a phase III trial of patients with community acquired pneumonia in those patients receiving rhG-CSF radiographic resolution of pneumonia was hastened, and the local (empyema) and systemic sequelae (ARDS, DIC) of pneumonie was decreased[50]. Although there is clinical evidence for the efficacy of rhG-CSF as anti-inflammatory agent, further studies are required to determine whether rhG-CSF significantly reduces morbidity, mortality, and duration of symptoms in patients with severe infections.

G-CSF and nitric oxide

Severe sepsis following bacterial inoculation is believed to be induced by the release of endotoxins, eg, LPS, and the concomitant activation of the cellular immune system. These events culminate in hemodynamic instability and a deterioration of respiratory functions. Secretion of pro-inflammatory mediators like TNF-α and IL-1β represent one potential mechanism of these deleterious processes. A number of studies indicate that in addition the production of nitric oxide (NO) is involved in the cytotoxic and tissue damaging effects of sepsis as well as other forms of shock[51,52]. NO is generated by several cell types by the conversion of *L*-arginine to *L*-citrulline, a reaction which is catalyzed by the enzyme nitric-oxide synthase (NOS)[53]. To date, three NOS isoforms have been cloned which are primarily separated to be either constitutive (bNOS, ecNOS) or inducible (iNOS)[54].

Although it is conceivable that NO derived from any of these isoforms triggers pathophysiological processes, it has been suggested that only iNOS-derived NO mediates the detrimental vascular changes that occur during shock[55]. In fact, NO may represent the final common pathway leading to the pathophysiological hypotensive reaction characteristic of shock. In this regard, inhibition of excessive NO synthesis may provide another explanation for the ameliorating anti-inflammatory effects of G-CSF observed during severe inflammatory processes.

In a recent study we could demonstrate that G-CSF suppresses the effects of TNF-α/IFN-γ on iNOS gene expression in a type II alveolar epithelial cell line *in vitro*. This was accompanied by a decreased synthesis of iNOS protein as well as NO production and release. Since iNOS mRNA half time did not differ between TNF-α/IFN-γ- and TNF-α/IFN-γ + G-CSF-treated cells, G-CSF exhibited its inhibitory effect most likely at the transcriptional level[56]. Follow-up studies have shown that G-CSF-induced suppression of iNOS gene expression and NO generation is not restricted to alveolar epithelial cells. G-CSF decreased IFN-γ/LPS mediated iNOS synthesis and NO release in vascular smooth muscle cells, too[57]. Summarizing data of these experiments are shown in Tab 1, Fig 2. Squadrito *et al*[38] could demonstrate a salutary effect of G-CSF in a rat model of splanchnic artery occlusion shock. Following reperfusion, G-CSF markedly improved survival rates of occlusion shocked rats. This non-septic model of shock is characterized by marked hypotension, hyporeactivity to catecholamines, and an increase in TNF-α plasma concentrations. The irreversible circulatory failure is probably the result of an increased NO production via activation of iNOS gene expression in vascular smooth muscle cells[58]. In fact, G-CSF restored the hyporeactivity to catecholamines in endothelium-denuded aortic rings from untreated rats subjected to splanchnic artery occlusion[38]. In addition, the authors investigated the potential effects of rhG-CSF on iNOS activity in an *in vitro* model using peritoneal rat macrophages. rhG-CSF applied 6h after LPS significantly blunted NO production by stimulated macrophages[38].

However, concerning the NO-inhibitory capacity of G-CSF, controversial results have been reported by Golab and coworkers[59]. They investigated the influence of G-CSF on tumor growth in an *in vivo* model of melanoma in mice. When given alone, G-CSF did not influence tumor growth, but strongly potentiated the anti-tumor activity of interleukin-12. *Ex vivo* assay for macrophage NO synthesis performed in cells obtained by peritoneal lavage revealed that G-CSF administration further augmented the IL-12-induced secretion of nitric oxide. However, in a second set of experiments, the same group described a spontaneous release of NO from peritoneal macrophages of melanoma-bearing mice, which could be significantly inhibited by G-CSF application[60].

Tab 1. Nitrite/nitrate levels measured as accumulated nitrite (nmol/10^6 cells) in cell-free culture supernatants following 24-h incubations.

	L2 cells	Vascular smooth muscle cells
Unstimulated controls	4.8 ± 0.3	3.2 ± 0.6
Granulocyte colony-stimulating factor (250 kU·L^{-1})	3.6 ± 0.3	4.0 ± 0.4
Respective stimulus (STIM) for inducible nitric-oxide synthase gene expression (see legend)	106.1 ± 2.5[b]	91.9 ± 5.8[b]
STIM plus granulocyte colony-stimulating factor (250 kU·L^{-1})	66.9 ± 1.6[bc]	72.8 ± 3.2[bc]

Data are expressed as means ± SEM, $n = 8$. The stimulus for inducible nitric-oxide synthase gene expression in L2 cells was tumor necrosis factor-α (500 kU·L^{-1}) + interferon-γ (100 kU·L^{-1}), the stimulus for inducible nitric-oxide synthase gene expression in vascular smooth muscle cells was interferon-γ (100 kU·L^{-1}) + *Escherichia coli*-derived lipopolysaccharide (0.5 mg·L^{-1}). [b]$P < 0.05$ as compared to unstimulated controls. [c]$P < 0.05$ as compared to the respective incubations without granulocye colony-stimulating factor.

Conclusions: A proposed scheme between the possible interactions of pro- and anti-inflammatory cytokines, NO and G-CSF during severe inflammation/infection are shown in Fig 3. Taken together, these data indicate that G-CSF exhibits anti-inflammatory properties which may prove to be beneficial in situations associated with an increased activity of the cellular immune system. Since the suppressive effects of G-CSF on the production of pro-inflammatory mediators like TNF-α and nitric oxide are most likely neither cell type nor tissue specific, it is conceivable that NO release induced by pro-inflammatory mediators can be

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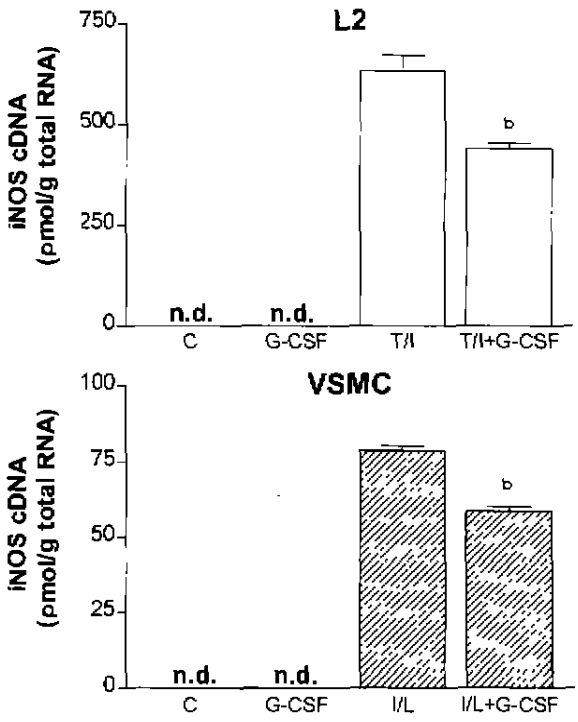

Fig 2. Quantitative analysis of inducible nitric oxides synthase (iNOS) gene expression detected as iNOS cDNA in type II-like alveolar epithelial cells (L2, upper part of the figure) and vascular smooth muscle cells (VSMC, lower part of the figure) following incubations with: 250 kU·L^{-1} granulocyte colony-stimulating factor (G-CSF), 500 kU·L^{-1} tumor necrosis factor-α/100 kU·L^{-1} interferon-γ (T/I), 500 kU·L^{-1} tumor necrosis factor-α/100 kU·L^{-1} interferon-γ + 250 kU·L^{-1} granulocyte colony-stimulating factor (T/I + G-CSF), 100 kU·L^{-1} interferon-γ/0.5 mg·L^{-1} lipopolysaccharide (I/L), and 100 kU·L^{-1} interferon-γ/0.5 mg·L^{-1} lipopolysaccharide + 250 kU·L^{-1} granulocyte colony-stimulating factor (I/L + G-CSF). C represents unstimulated controls. Data are given as means ± SEM (n = 5). $^bP < 0.05$ as compared to the respective incubations without G-CSF.

reduced by G-CSF in various organ systems and in different forms of shock. In this context, G-CSF might represent a counterregulatory mechanism directed against a downstream oriented inflammatory response to infection. Therefore, the investigation of G-CSF in the prophylaxis of nonneutropenic infections, sepsis, and other severe inflammatory disorders seems reasonable.

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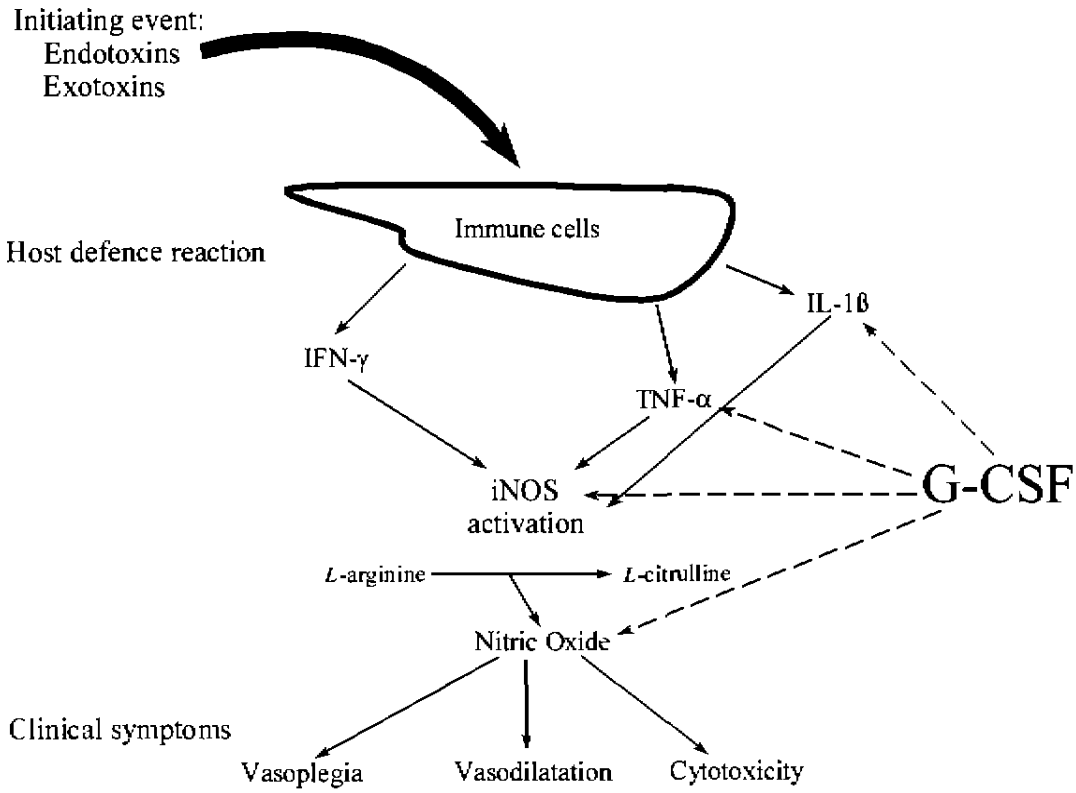

Fig 3. Possible pathway of pro- and anti-inflammatory cascades during septic shock. Solid lines indicate the pro-inflammatory pathways, dashed lines indicate the possible anti-inflammatory mechanisms of granulocyte colony-stimulating factor (G-CSF). IFN-γ: interferon-γ; IL-1β: interleukin-1β; iNOS: inducible nitric-oxide synthase; TNF-α: tumor necrosis factor-α.

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关键词 粒细胞集落刺激因子; 细胞因子类; 感染; 炎症; 脓毒症; 一氧化氮合酶

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