Impairment of Growth of *Listeria monocytogenes* in THP-1 Macrophages by Granulocyte Macrophage Colony-Stimulating Factor: Release of Tumor Necrosis Factor–α and Nitric Oxide

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Background. *Listeria monocytogenes* tends to survive in phagocytes. Granulocyte macrophage colony-stimulating factor (GM-CSF) protects mice against *L. monocytogenes* infection, and mice knocked out for the GM-CSF gene are more susceptible to these infections.

Methods. THP-1 cells were used to characterize the GM-CSF receptor (binding isotherms; STAT5 phosphorylation), measure the intracellular growth of *L. monocytogenes* (5 h after phagocytosis), examine the influence of a 24-h incubation with GM-CSF before infection, measure the production of tumor necrosis factor (TNF)–α and the expression of nitric oxide synthase (iNOS), and evaluate the influence of anti–GM-CSF receptor (GM-CSFRα) and anti—TNF-α antibodies and the addition of N_{ω}-nitro-L-arginine methyl ester (L-NAME) and catalase.

Results. THP-1 cells display functional GM-CSFRα. GM-CSF impairs the intracellular growth of *L. monocytogenes* to ~65% of its value in unstimulated cells. This effect is abolished by anti–GM-CSFRα, anti–TNF-α antibodies, and catalase (and, to a lesser extent, by L-NAME). GM-CSF stimulates the release of TNF-α and the expression of iNOS. TNF-α added to unstimulated cells (even in large amounts) does not fully reproduce the impairment in the growth of *L. monocytogenes* caused by GM-CSF.

Conclusions. GM-CSF impairs the intracellular growth of *L. monocytogenes* by a synergistic action of the GM-CSF–triggered release of autocrine TNF-α and hydrogen peroxide and the production of NO (associated with the stimulation of the expression of iNOS).

Listeria monocytogenes is a facultative intracellular foodborne pathogen that causes life-threatening infections that mostly affect immunocompromised patients [1, 2]. These infections are often associated with a high rate of recurrence and dissemination, which has been related to the lack of eradication of intracellular forms, particularly those hosted in phagocytes [3]. The results of previous studies have shown that interferon (IFN)–γ promotes the clearance of *L. monocytogenes* in vivo [4], and the results of in vitro studies have suggested that this effect could be mediated by a containment of *L. monocytogenes* in phagosomes [5] and the release of nitric oxide (NO) and hydrogen peroxide [6]. Granulocyte macrophage colony-stimulating factor (GM-CSF) is another cytokine that protects mice against *L. monocytogenes* infection [7]. It has also been shown that mice knocked out for the GM-CSF gene are more susceptible to *L. monocytogenes* infection [8]. GM-CSF stimulates the production of tumor necrosis factor (TNF)–α [9] and enhances the oxidative burst that occurs after phagocytosis [10]. The aim of the present study was to establish whether GM-CSF has an inhibitory effect on the intracellular growth of *L. monocytogenes* in macrophages and to try to unravel the mechanism of this effect in relation to the release of TNF-α

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and the expression of inducible NO synthase (iNOS). For this purpose, we used a previously established model of human macrophages (THP-1 cells). These cells are highly permissive for intracellular infection when they are unstimulated [11–15], but they can impair the intracellular growth of *L. monocytogenes* when they are activated by IFN-γ [14]. Our results show that GM-CSF also impairs the intracellular multiplication of *L. monocytogenes.* This effect is related to the binding of GM-CSF to specific surface receptors and is mediated by a synergistic action of GM-CSF–triggered autocrine secretion of TNF-α and the GM-CSF–stimulated expression of iNOS.

MATERIALS AND METHODS

Cytokines, antibodies, and main reagents. Recombinant human GM-CSF, recombinant human TNF-α, and monoclonal anti–human TNF-α antibodies were purchased from R&D Systems, and recombinant human IFN-γ was purchased from Roche Diagnostics. Monoclonal anti–GM-CSF receptor–α (GM-CSFRα) antibodies were purchased from Maine Biotechnology Services. All cytokines were aliquoted in sterile PBS supplemented with 0.1% bovine serum albumin (BSA), stored at -20°C, and thawed immediately before use. N_{ω}-nitro-L-arginine methyl ester (L-NAME) and catalase were purchased from Sigma-Aldrich. Gentamicin was kindly donated by Shering-Plough Belgium in the form of Geomycin (the brand name of the product registered for clinical use in Belgium). Cell-culture media and serum were purchased from Gibco Biocult (Life Science Technologies). Unless stated otherwise, all other reagents were purchased from E. Merck.

Cell cultures. All experiments were performed with THP-1 cells (ATCC TIB-202), a human myelomonocytic cell line that displays macrophage-like activity [16]. These cells were maintained as a loose suspension in RPMI 1640 medium supplemented with 10% decomplemented fetal calf serum (FCS) and 2 mmol L-glutamine/L in an atmosphere of 95% air/5% CO_2. Cells were subcultured every third day by gentle shaking, followed by pelleting and reseeding at a density of $\sim 3 \times 10^5$ cells/mL. The cultures were used after 2 days, when they had reached a density of $\sim 0.5–1 \times 10^6$ cells/mL.

Characterization of GM-CSF receptors. Binding assays were performed by exposing 10^7 cells/mL at 4°C for 2 h to increasing concentrations of [^{125}I]-GM-CSF (specific radioactivity, 47.8 TBq/mmol; NEN Perkin Elmer Life Science) in RPMI 1640 medium supplemented with 2% FCS. After incubation, cells were washed 4 times with ice-cold PBS supplemented with 2% FCS, and cell-associated radioactivity was thereafter determined by γ-scintillation counting. Nonspecific binding was determined by repeating these experiments in the presence of a 100-fold excess of unlabeled GM-CSF. Specific binding was determined by subtraction of the nonspecific binding from the total binding. Binding parameters were determined by nonlinear regression (1 site-binding hyperbola). Phosphorylation of the transcription activator of GM-CSF (STAT5) [17, 18] was assessed by Western Blot analysis, by use of specific anti-phosphorylated STAT5 antibodies (see details below).

***Evaluation of the influence of GM-CSF on the intracellular growth of* L. monocytogenes.** Unless stated otherwise, the following protocol was adopted. Cells were exposed to GM-CSF for 24 h at 37°C, washed in prewarmed sterile PBS, and then challenged with *L. monocytogenes* infection (5 h of intracellular growth) in the absence of GM-CSF, according to a method described in detail elsewhere [19]. In brief, 10^6 cells were exposed to a fresh inoculum of 5×10^6 cfu of *L. monocytogenes* (hemolysin-producing strain EGD, serotype 1/2a) in a volume of 2 mL, for 1 h at 37°C. Nonphagocytosed and non–firmly adherent bacteria were removed by 4 successive centrifugations and washing in prewarmed sterile PBS. Cells were thereafter placed in fresh medium and collected immediately (time 0) or cultured for 5 h. As has been shown elsewhere [14, 19], no extracellular growth of *L. monocytogenes* occurs during this time frame. Cells were harvested by centrifugation, washed with ice-cold sterile PBS, and lysed in distilled water. Samples were then plated on tryptic soy agar after appropriate dilutions, for determination of the number of viable bacteria by counting colony-forming units after 24 h of incubation at 37°C, using a Gel Doc 2000 apparatus (Bio-Rad Laboratories) operated with Quantity One software (Bio-Rad Laboratories). The actual number of colonies counted was typically between 200 and 800 and was never <80. The number of colony-forming units in each sample was related to its cell protein content, and results were expressed as growth index by calculating the ratio of the number of colony-forming units per milligram of protein observed in samples collected 5 h after phagocytosis to the number observed after phagocytosis. The absence of extracellular bacteria was ascertained by plating aliquots of the culture medium collected at the end of the incubation. No bacterial growth was detected after 48 h at 37°C.

Measurement of TNF-α production. This was performed by use of an ELISA with OptEIA Set human TNF-α (Pharmingen), by use of a standard protocol. In brief, supernatants collected by centrifugation of THP-1 cells for 24 h (with or without GM-CSF) were added to 96-wells plates coated with mouse anti–human TNF-α antibody (dilution, 1:500). Results were revealed by using biotinylated mouse anti–human TNF-α antibody (dilution, 1:500) and avidin–horseradish peroxidase (HRP; dilution, 1:250), followed by 400 mg/L of *o*-phenylenediamine and 10% urea peroxide in citrate buffer (0.1 mol/L, pH 5). The reaction was stopped after 30 min in the dark with 2 *N* H_2SO_4. Optical densities were read with a Novapath plate reader (Bio-Rad Laboratories), by use of a 490- and 650-nm filter for background.

Western Blot analyses (phospho-STAT5, iNOS, and actin). Cells were collected by centrifugation, resuspended, and lysed in O'Farrel 1× sample buffer (Laemmli sample buffer; Bio-Rad Laboratories, β-mercaptoethanol and water in a ratio of 95:5:100) at a concentration of 10^6 cells/mL. Lysates were then subjected to SDS-PAGE and immunoblotted with appropriate antibodies: rabbit monoclonal anti–human phosphoSTAT5 (Cell Signaling Technology; diluted 1:500 in 0.8% BSA), iNOS, rabbit polyclonal antihuman NOS 2 (Santa Cruz Biotechnology; diluted 1:500 in 0.8% BSA), actin, and mouse monoclonal anti–human β-actin (Sigma-Aldrich, diluted 1:1000 in 0.8% BSA). The membranes were applied to Clear Blue X-ray films (CL-Xposure film; Pierce Biotechnology), and the bands were revealed with goat anti-rabbit (phospho-STAT5 and iNOS) or goat anti-mouse (actin) polyclonal antibodies (diluted 1:500 in 0.8% BSA) coupled to HRP (1:20,000; Transduction Laboratories), by use of Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology). The films were then developed and scanned using an Image Station 440 CF (Kodak Digital Sciences) and DS-1-D software (Eastman Kodak). The apparent molecular weight of the proteins of interest was estimated using standards of known molecular weight ran in parallel with the samples (BenchMark Pre-Stained Protein Ladder; Invitrogen).

Biochemical assays. The protein content of the samples was assayed by use of the Folin-Ciocalteu/biuret method [20], and NO release was measured by use of the nitrite/nitrate colorimetric method and a commercial kit (Roche Diagnostics).

Statistical analysis. Curve-fitting analyses were performed with Prism software (version 2.01; GraphPad Software). The statistical significance of differences between means was determined using Student's *t* test.

RESULTS

Characterization of GM-CSF receptors. In a first series of experiments, we tested for the presence and functionality of high-affinity GM-CSF receptors on THP-1 cells. As shown in figure 1, the binding of [^{125}I]-GM-CSF was saturable, with an apparent K_D = 13.87 pmol/L (0.2 ng/mL) and an estimated maximum binding capacity of ~100 molecules/cell (under the assumption of monovalent receptor-ligand interaction). The phosphorylation of STAT5 (the transcription activator of GM-CSF) [17, 18] was then examined by Western Blot analysis of lysates of cells incubated with GM-CSF (5–40 ng/mL for 24 h at 37°C), and results were revealed by use of an antibody directed against the phosphorylated STAT5. The upper inset of figure 1 shows a definite reaction for a protein with an estimated molecular weight of 90 kDa, which is typical of STAT5.

***Influence of GM-CSF on the intracellular growth of* L. monocytogenes.** In our model, *L. monocytogenes* achieved an ~10-fold intracellular growth within 5 h after phagocytosis,

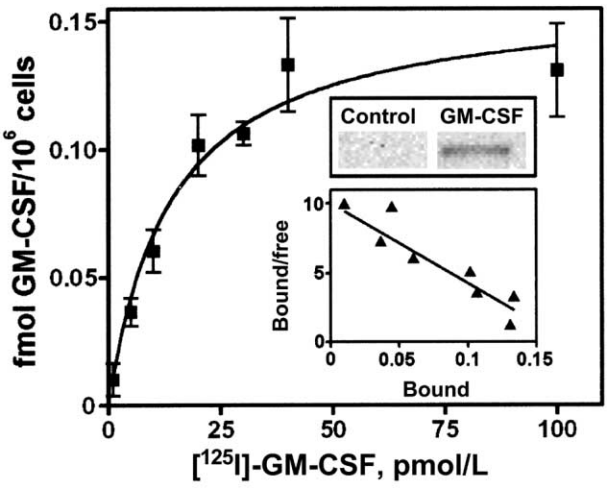

Figure 1. Characterization of granulocyte macrophage colony-stimulating factor (GM-CSF) receptors in THP-1 cells. *Main graph,* binding of [^{125}I]-GM-CSF to cells at 4°C (2 h). Nonspecific binding (measured in the presence of a 100-fold excess of unlabeled GM-CSF) has been subtracted. Data are means of 3 determinations ± SD. These experiments were performed twice, with very similar results. *Lower inset,* Same values, presented as a Scatchard plot (R^2 = .953). *Upper inset,* phosphorylation of STAT5 in cells exposed to GM-CSF. Cells were incubated for 24 h at concentrations of 5–40 ng/mL/h at 37°C with 5–40 ng/mL GM-CSF (only the result obtained at 20 ng/mL is shown, because this concentration was used for most of the subsequent experiments). Cell lysates were analyzed by Western blot, using an antiphospho-STAT5 antibody. The migration distance of the band corresponds to an estimated molecular weight of 90 kDa.

compared with the initial inoculum [19]. Figure 2*A* shows that this growth was impaired to ~65% of the value of controls when cells had been preincubated for 24 h with 20 ng/mL GM-CSF. In a next series of experiments, we examined the concentration dependency of the impairment of the growth of *L. monocytogenes* caused by GM-CSF. Figure 2*B* shows that a marked decrease in growth was observed once the concentration of GM-CSF was >5 ng/mL and reached its maximal effect at ~20 ng/mL.

To ascertain that GM-CSF acted on the host cell and not directly on bacteria, we first tested that this cytokine had no direct effect on the growth of *L. monocytogenes* in broth. No influence was seen on growth curves, for concentrations up to 200 ng/mL. We then measured the capacity of THP-1 cells to phagocytose *L. monocytogenes* and found no significant differences in initial inoculum between control and GM-CSF–exposed cells. To further discount an effect on extracellular bacteria, the experiments described in figure 2*A* were repeated using gentamicin (18 mg/L; 20× the MIC, added during the 5 h after phagocytosis incubation). As shown in table 1, gentamicin caused an apparent reduction in growth of ~15%, compared with controls (this effect has been observed before and was ascribed to the killing activity of gentamicin toward adherent, but not phagocytosed, bacteria and bacteria that re-

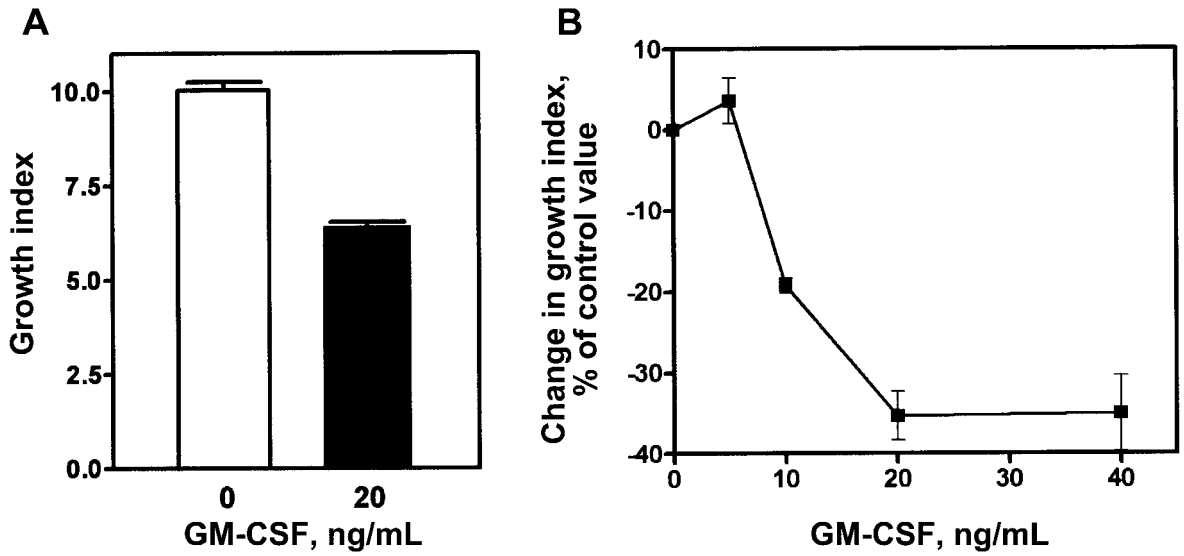

Figure 2. Influence of granulocyte macrophage colony-stimulating factor (GM-CSF) on the intracellular growth of *Listeria monocytogenes* in THP-1 macrophages. *A,* Control cells (no GM-CSF) or cells exposed to GM-CSF, which were incubated for 24 h at 37°C before infection with *L. monocytogenes* and further intracellular growth (5 h) in the absence of GM-CSF. The growth index is defined as the ratio of the no. of colony-forming units (cfu)/mg cell protein collected from cells at the end of the 5-h incubation time to the no. of cfu/mg cell protein observed after phagocytosis and washing of the extracellular bacteria (time, 0 h). Each value is the mean (± SE) of 6 independent experiments, with each sample assayed in triplicate. *B,* Cells exposed to increasing concentrations of GM-CSF (24 h, 37°C) before being challenged for *L. monocytogenes* infection, as in the experiments shown in panel *A.* Each value is the mean (± SE) of 3 independent experiments, with each sample being assayed in triplicate.

mained in phagosomes [19]; this decrease was indeed observed during the first 1 h of incubation, after which bacterial growth proceeded at the same rate as in cells not exposed to gentamicin). Under these conditions, pretreatment of cells with GM-CSF reduced the growth of *L. monocytogenes* to ~57% of the value observed without pretreatment, versus 66% for cells not exposed to gentamicin. Although this difference was not statistically significant, it illustrates that the effect of GM-CSF is probably directed against intracellular bacteria.

To establish whether the impairment of the growth of *L. monocytogenes* caused by GM-CSF was related to its binding to its receptor, these experiments were repeated in the presence of monoclonal blocking antibodies raised against the α-subunit of GM-CSFRα (2.5 μg/mL; this concentration was ~2.5 times the concentration needed to neutralize all GM-CSF receptors present in the system, on the basis of the titration of the batch of antibody used in these studies). Antibodies were added to the culture medium before the addition of GM-CSF and were maintained until GM-CSF was washed off and cells were challenged by infection with *L. monocytogenes.* Table 1 shows that the impairment of intracellular bacterial growth caused by GM-CSF was completely abolished under these conditions. We tested whether the addition of gentamicin (18 mg/L) modified the neutralizing effect of the anti–GM-CSFRα antibodies; to ensure that they did not act in an unspecific fashion on cell defense mechanisms or by a mere toxic effect, we measured the effect of IFN-γ on the intracellular growth of *L. monocytogenes* in the presence and absence of these antibodies. In both cases, the growth of *L. monocytogenes* was inhibited to ~20% of the value observed in unstimulated cells, which is similar to what has been observed elsewhere in the same cells [14].

Role of TNF-α. GM-CSF is known to induce the production of TNF-α in macrophages [9]. We therefore measured its release from THP-1 cells on exposure to GM-CSF, at increasing concentrations, for 24 h. As shown in figure 3*A*, a detectable level of release was noted at a concentration of 5 ng/mL, but it increased markedly thereafter, with a tendency to reach a plateau at ~2.5 ng/mL for cells incubated with $\geq$20 ng/mL GM-CSF. The impairment of the growth of *L. monocytogenes* was correlated with the level of the production of TNF-α (figure 3*A*, inset). We then examined the influence of neutralizing monoclonal antibodies raised against human TNF-α. These antibodies, added in an amount sufficient to neutralize ~10 ng/mL TNF-α, were maintained during the entire exposure of the cell to GM-CSF. As shown in figure 3*B,* although GM-CSF caused a significant decrease in bacterial growth ($P < .05$), the addition of anti–TNF-α antibodies completely abolished this effect. These antibodies had no effect on the growth of *L. monocytogenes* in cells that were not exposed to GM-CSF. To further rule out any unspecific action of these anti–TNF-α antibodies, we examined their influence on the GM-CSF–triggered phosphorylation of STAT5 and the impairment of the growth of *L. monocytogenes* induced by IFN-γ. No effect was seen in either case.

To test whether the secretion of TNF-α was the only sufficient

Table 1. Influence of gentamicin and anti–granulocyte macrophage colony-stimulating factor receptor (GM-CSFRα) antibodies on the activity of GM-CSF toward the intracellular growth of *Listeria monocytogenes*.

	Growth index[a]	
	Control	GM-CSF
No addition	10.1 ± 1.2[a]	6.7 ± 0.7[c]
Gentamicin (18 μg/mL)[b]	8.5 ± 0.9[b]	4.9 ± 0.3[d]
Anti—GM-CSFRα antibodies[c]	10.0 ± 0.8[a]	10.6 ± 1.6[a]

NOTE. Nos. with different letters are significantly different from each other ($P < .05$).

[a] No. of cfu/mg protein at 5 h/no. of cfu at 0 h (after phagocytosis).

[b] 10× MIC; added after phagocytosis and during the 5-h incubation time used to evaluate the intracellular growth of *L. monocytogenes*.

[c] Added during the exposure of cells to GM-CSF (24 h before infection).

mediator to cause the impairment of the intracellular growth of *L. monocytogenes* on treatment of the cells with GM-CSF, we examined the influence of exogenous TNF-α (10 ng/mL) maintained in the presence of the cells for 24 h at 37°C before challenging them with *L. monocytogenes*. As shown in figure 3*B*, TNF-α alone had only a modest, nonsignificant effect that did not fully reproduce the effect observed with GM-CSF.

Role of NO and hydrogen peroxide. Murine macrophages are known to produce large quantities of NO and hydrogen peroxide on stimulation with GM-CSF [10, 21]. The results of Western blot analysis (figure 4) showed that the expression of iNOS was markedly induced in THP-1 cells after 24 h of treatment with GM-CSF, without the addition of anti–TNF-α antibodies (which demonstrates that it was not primarily mediated by the release of TNF-α; we also tested whether the addition of L-NAME was without influence on this expression; data not shown). In parallel, we measured the production of NO in cells treated with GM-CSF (using the nitrite/nitrate method) and found a marked increase in its production, compared with controls. This production was abolished in the presence of L-NAME (400 μmol/L; data not shown). We then examined the influence of L-NAME, catalase, and the combination of both on the impairment of the growth of *L. monocytogenes* induced by GM-CSF. As shown in figure 5, L-NAME and catalase, when maintained in contact with the cells for 24 h at 37°C together with GM-CSF, not only abolished the impairing effect of GM-CSF on the intracellular growth of *L. monocytogenes* in cells treated with GM-CSF alone ($P < .05$) but actually significantly stimulated this growth (~28% more than in controls). A similar increase was seen in cells incubated with GM-CSF and catalase alone, which indicates that the production of hydrogen peroxide was probably a main factor that contributed to the impairment of the growth of *L. monocytogenes* in GM-CSF–stimulated THP-1 cells. L-NAME alone was also effective in abolishing the impairing effect of GM-CSF and in stimulating the growth of *L. monocy-*

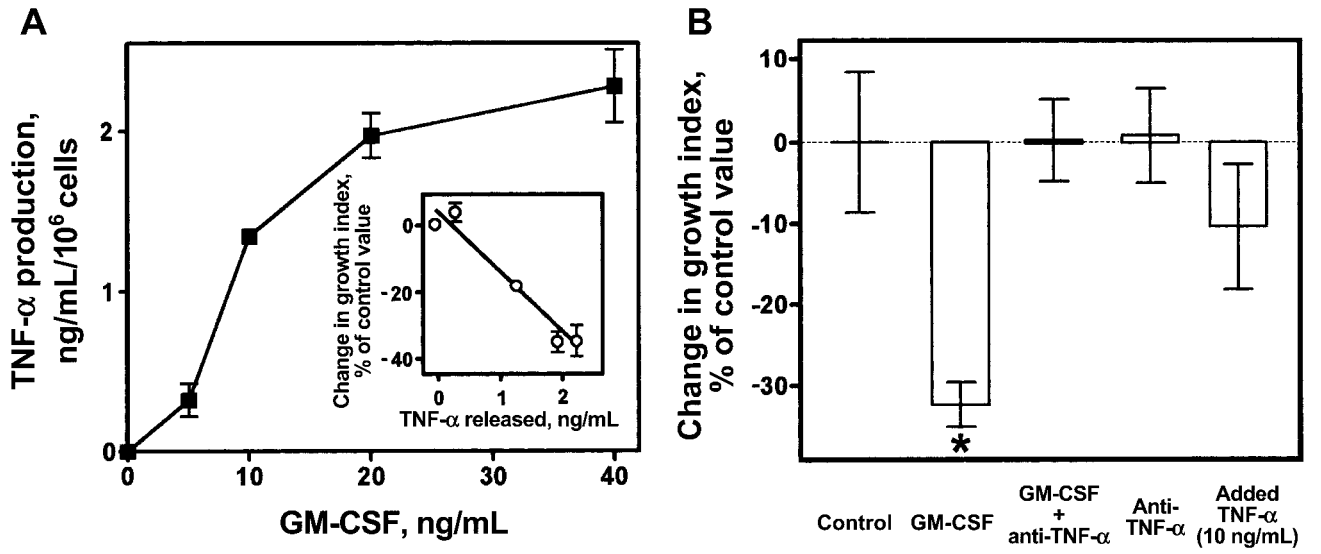

Figure 3. Secretion and role of tumor necrosis factor (TNF)–α in the impairment of growth of *Listeria monocytogenes* induced by granulocyte macrophage colony-stimulating factor (GM-CSF) in THP-1 cells. *A,* Release of TNF-α in the culture medium after a 24-h incubation at 37°C in the presence of increasing concentrations of GM-CSF. Data are means (± SD) of 3 determinations. The experiment was performed twice, with very similar results. *Inset,* Correlation between the release of TNF-α on exposure to GM-CSF (main graph) and impairment of the growth of *L. monocytogenes* (figure 2*B*). *B,* Influence of GM-CSF (20 ng/mL), GM-CSF (20 ng/mL) plus anti–TNF-α antibodies (2 μg/mL), anti–TNF-α antibodies (2 μg/mL), or added TNF-α (10 ng/mL) on the intracellular growth of *L. monocytogenes*. Experiments were conducted as described in figure 2 (see caption for the definition of the growth index). Data are means (± SD) of 3 determinations. Differences between groups were assessed by use of Student's *t* test (*$P < .05$). These experiments were performed twice, with very similar results.

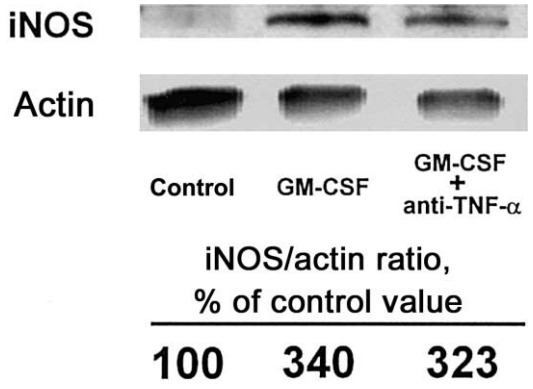

Figure 4. Expression of inducible nitric oxide synthase (iNOS) and of actin in control THP-1 cells and in cells exposed for 24 h at 37°C to granulocyte macrophage colony-stimulating factor (GM-CSF; 20 ng/mL) or GM-CSF (20 ng/mL) plus anti–tumor necrosis factor (TNF)–α antibodies (2 μg/mL), as evidenced by Western blot analysis of cell lysates using anti iNOS and anti–actin antibodies, respectively. The migration distance of the bands corresponds to an estimated molecular weight of 118 (iNOS) and 47 (actin) kDa, respectively. The figures show the results of a photodensitometric scanning of the films in which the ratio of the iNOS to actin bands in each sample is expressed as the percentage of the value found in control cells.

togenes, but to a lesser extent. The addition of L-NAME, catalase, or both, without GM-CSF, had the same effect as that observed in the presence of GM-CSF (data not shown).

DISCUSSION

The present study has provided direct evidence that GM-CSF impairs the intracellular growth of *L. monocytogenes* in THP-1 cells and that this effect is mediated by the binding of this cytokine to its receptor and by a GM-CSF–triggered release of TNF-α and oxidant species (nitrogen oxide and hydrogen peroxide) related to the expression of iNOS. THP-1 macrophage-like cells were chosen because they are largely unable to kill *L. monocytogenes,* in contrast to what is observed with resident macrophages or monocytes [22, 23]. This offered the possibility to quantify the influence of GM-CSF independently of the other mechanisms of cell defense, a point that may need to be taken into account to explain the discordant results concerning the action of GM-CSF on *Listeria* infection [24]. Conversely, it must be stressed that THP-1 cells are undifferentiated, nonadherent cells that may, therefore, not represent fully functional macrophages as they exist in vivo. Yet we have shown here that THP-1 cells have a basal production of oxidant species that limits the inhibition of the growth of *L. monocytogenes.* Moreover, these cells respond to GM-CSF to further limit this growth and have successfully been used previously to examine the mechanism of the protective effect exerted by IFN-γ against *L. monocytogenes* infection and to analyze its cooperation with antibiotics for the eradication of *L. monocytogenes* from these cells [6, 14].

The impairment of intracellular growth observed in THP-1 cells pretreated with GM-CSF can reasonably be attributed to an effect toward the intracellular bacteria that is related to the activation of a specific surface receptor. First, we have shown that THP-1 cells display high-affinity receptors for GM-CSF, with binding parameters to similar those found in other macrophages [25, 26] and with evidence of functionality (as assessed by the demonstration of the ligand-triggered phosphorylation of STAT5) [17, 18]. Second, all influence of GM-CSF on the growth of *L. monocytogenes* is suppressed when activation takes

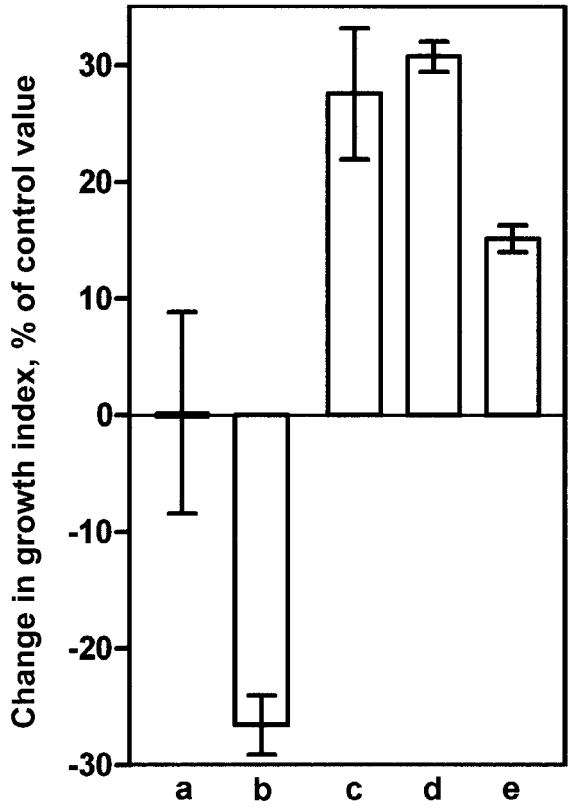

Figure 5. Role of nitric oxide and hydrogen peroxide in the impairment of the growth of *Listeria monocytogenes* induced by granulocyte macrophage colony-stimulating factor (GM-CSF) in THP-1 cells: influence of GM-CSF (20 ng/mL), GM-CSF (20 ng/mL) plus N_{ω}-nitro-L-arginine methyl ester (L-NAME; 400 μmol/L), and catalase (1500 U/mL), and only L-NAME (400 μmol/L) and catalase (1500 U/mL), each in combination with GM-CSF (20 ng/mL). Cells incubated with L-NAME and catalase but without GM-CSF showed a bacterial growth similar to that observed when they were combined with GM-CSF (data not shown). Experiments were conducted as described in figure 2 (see caption for the definition of the growth index). Data are means ($\pm$ SD) of pooled data from 2 series of experiments (with 3 determinations in each series). Statistical analysis (Student's *t* test with $P < .05$) showed that a, b, c, and e are different from one another but not c and d. a, Control; b, GM-CSF; c, GM-CSF, catalase, and L-NAME; d, GM-CSF and catalase; and e, GM-CSF and L-NAME.

place in the presence of an anti–receptor-blocking antibody. This antibody did not impair the IFN-γ–dependent protective effect and could, therefore, not act in an unspecific fashion or by a global toxic effect. Third, there was no evidence of a direct toxic effect of GM-CSF on bacteria, as evidenced by the lack of effect of GM-CSF on bacterial growth in broth and the additive effects of treatment with GM-CSF (before phagocytosis) and gentamicin (added after phagocytosis). The latter finding strongly suggests that GM-CSF and gentamicin act on distinct pools of bacteria—namely, the truly intracellular ones for GM-CSF and the adherent but not phagocytosed organisms, as well as those remaining in phagosomes for gentamicin [19]. This also allows us to discount an effect of GM-CSF on bacterial adherence and an ensuing slowing down of the internalization of *L. monocytogenes*—in that case, GM-CSF and gentamicin should not have additive effects, because their action would be exerted against the same pool of extracellular bacteria. Fourth, we discounted a potential effect of GM-CSF on the phagocytosis of *L. monocytogenes* in our model. This control was important, because early studies had shown that macrophages obtained from transgenic mice that produce high levels of GM-CSF phagocytose more actively than cells from normal littermates while being no more efficient at lysing intracellular bacteria [27]. It is therefore important to underline that the phagocytosis of *L. monocytogenes* and its subsequent intracellular growth were studied here in the absence of GM-CSF during this process. This experimental design was specifically meant to examine the long-term result of the activation of the cells by GM-CSF on the handling of *L. monocytogenes,* and not a direct effect of GM-CSF on the bacteria (whether making them more fragile or enhancing their level of phagocytosis).

The initial role of the binding of GM-CSF to its receptor in the subsequent impairment of the growth of *L. monocytogenes* could be disputed on the basis that a significant protective effect is only seen at concentrations (≥10 ng/mL) that are far above the saturation point of the putative receptor of [^{125}I]-labeled GM-CSF. A plausible explanation could be that the low number of receptors for GM-CSF on THP-1 cells makes them relatively insensitive to the action of this cytokine, which therefore requires the full occupancy of the receptors to yield a significant protective effect. Future experiments with cells overexpressing the GM-CSF receptor could be useful in this context.

The role of TNF-α as a mediator of GM-CSF, for impairing the intracellular growth of *L. monocytogenes* appears clearly from the observations: the production of TNF-α after exposure of the cells to GM-CSF proceeds quantitatively, almost in parallel with its effect on bacterial growth, and the neutralization of TNF-α suppresses all response to treatment with GM-CSF. The simple fact, however, that the addition of TNF-α to cells not exposed to GM-CSF does not allow them to reproduce the action of GM-CSF also indicates that TNF-α alone is insufficient to bring about a significant impairment of the growth of *L. monocytogenes.* Our current hypothesis is, therefore, that TNF-α produced by cells on stimulation with GM-CSF acts through a NO-independent pathway but that neither autocrine TNF-α nor induced free radicals are able to control the intracellular growth of *L. monocytogenes* by themselves in THP-1 macrophages. The necessity for TNF-α to be combined with other factors to exert a significant effect on the growth of *L. monocytogenes* has already been observed in IFN-γ [5]. In parallel, TNF-α can limit the growth of *L. monocytogenes* in macrophages in a NO-independent manner [28], which suggests the existence of multiple mechanisms. Finally, although very large amount of nitrosative species may be needed to obtain a complete inhibition of the intracellular growth of *L. monocytogenes,* macrophages stimulated simultaneously by IFN-γ, TNF-α, and lipopolysaccharide are still able to reduce intracellular growth in the presence of iNOS inhibitors, which demonstrates the involvement of both NO-dependent and NO-independent pathways for the control of bacterial growth [29].

Murine macrophages are known to produce large concentrations of NO and hydrogen peroxide after stimulation with GM-CSF [10, 21], and resident peritoneal cell populations from GM-CSF$^{-/-}$ mice show a reduced production of NO [30]. We have shown here that THP-1 cells also respond to GM-CSF by an increase in the production of NO, and we have presented evidence of a GM-CSF–dependent expression of iNOS in these cells. The data also show that the expression of iNOS and the production of TNF-α are 2 independent consequences of the exposure of THP-1 cells to GM-CSF, because the expression of iNOS was not affected by the presence of antibodies raised against TNF-α during exposure to GM-CSF. In parallel, we have shown that the production of hydrogen peroxide is critical in the impairment of the growth of *L. monocytogenes* that is brought about by GM-CSF, because this effect of GM-CSF was entirely defeated by the addition of catalase. NO, however, should also play a role, because the addition of L-NAME was also effective. Incidentally, these results also show that unstimulated THP-1 cells were capable of a basal production of NO and hydrogen peroxide in response to infection: bacterial growth was more extensive in the presence of L-NAME and catalase (without GM-CSF) than in control cells. GM-CSF appears, therefore, to be an add-on factor in this context. We do not know, however, whether the GM-CSF–triggered increased expression of iNOS and production of NO (and, most likely also, of hydrogen peroxide) is sufficient to impair the growth of *L. monocytogenes* or whether TNF-α must also be present. The results of previous studies have shown that TNF-α triggers macrophages to produce large amounts of reactive nitrogen intermediates but that the complete inhibition of this production does not decrease cell bactericidal activity [28], which suggests a direct effect of TNF-α on bacterial growth. Conversely, no additional factor besides NO and hydrogen perox-

ide seems to be necessary to severely limit the growth of *L. monocytogenes* in THP-1 cells pretreated with IFN-γ [6]. The latter, however, not only triggers the production of nitrogen and oxygen reactive species but also prevents the migration of *L. monocytogenes* from phagosomes to the cytosol, where the bacteria will actively multiply [5]. We do not know how GM-CSF influences the subcellular distribution of *L. monocytogenes* in THP-1 cells nor how a synergy among TNF-α, NO, and hydrogen peroxide could be important in this context. These critical issues will need to be examined in future experiments.

In spite of these uncertainties, our results already provide interesting perspectives in 2 complementary directions. First, they may help us to understand how GM-CSF affords protection of the immunocompetent host against infection by *L. monocytogenes,* as has been demonstrated in animals [7, 8]. In a broader context, GM-CSF could therefore be viewed as participating in the general control of the *L. monocytogenes* infection by the immunocompetent host, making the bacteria less able to survive intracellularly, as has also been observed with IFN-γ [5, 6]. This potential role of GM-CSF, in combination with other cytokines, for increasing the antibacterial competence of macrophages has already been advocated [22]. More specifically, GM-CSF could also cooperate for defense against *Listeria* infection with other proinflammatory cytokines, such as interleukin (IL)–12 [31, 32] and IL-18 [33, 34]. Intriguingly, the administration of a traditional Chinese medicine that protects against *Listeria* infection in mice has been shown to trigger the production of IL-1, IL-6, and GM-CSF [35]. Conversely, it could be of interest to monitor the production of GM-CSF in response to an *L. monocytogenes* infection and to examine to what extent variations under specific conditions and/or among patients could be correlated to commensurate changes in risk factors. Second, our data may suggest the use of GM-CSF as a pharmacological agent for the control of *Listeria* infection in patients who are at risk. It could be argued that a protective effect of GM-CSF could only be observed at concentrations far above those observed physiologically in humans, even during infection and/or after the administration of endotoxin [36]. Yet the administration of GM-CSF for postchemotherapy bone-marrow recovery at conventional doses allows it to reach concentrations up to 5 ng/mL, and higher doses appear to be well tolerated [37]. Animal and exploratory clinical trials with GM-CSF may therefore be warranted.

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References

1. Vazquez-Boland JA, Kuhn M, Berche P, et al. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev **2001**; 14:584–640.
2. Wing EJ, Gregory SH. *Listeria monocytogenes:* clinical and experimental update. J Infect Dis **2002**; 185(Suppl 1):S18–24.
3. Drevets DA. Dissemination of *Listeria monocytogenes* by infected phagocytes. Infect Immun **1999**; 67:3512–7.
4. Buchmeier NA, Schreiber RD. Requirement of endogenous interferon-gamma production for resolution of *Listeria monocytogenes* infection. Proc Natl Acad Sci USA **1985**; 82:7404–8.
5. Portnoy DA, Schreiber RD, Connelly P, Tilney LG. Gamma interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. J Exp Med **1989**; 170:2141–6.
6. Ouadrhiri Y, Scorneaux B, Sibille Y, Tulkens PM. Mechanism of the intracellular killing and modulation of antibiotic susceptibility of *Listeria monocytogenes* in THP-1 macrophages activated by gamma interferon. Antimicrob Agents Chemother **1999**; 43:1242–51.
7. Magee DM, Wing EJ. Secretion of colony-stimulating factors by T cell clones: role in adoptive protection against *Listeria monocytogenes.* J Immunol **1989**; 143:2336–41.
8. Zhan Y, Lieschke GJ, Grail D, Dunn AR, Cheers C. Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of *Listeria monocytogenes*–infected mice. Blood **1998**; 91:863–9.
9. Chantry D, Turner M, Brennan F, Kingsbury A, Feldmann M. Granulocyte-macrophage colony stimulating factor induces both HLA-DR expression and cytokine production by human monocytes. Cytokine **1990**; 2:60–7.
10. Bermudez LE. Differential mechanisms of intracellular killing of *Mycobacterium avium* and *Listeria monocytogenes* by activated human and murine macrophages: the role of nitric oxide. Clin Exp Immunol **1993**; 91:277–81.
11. Garcia RC, Banfi E, Pittis MG. Infection of macrophage-like THP-1 cells with *Mycobacterium avium* results in a decrease in their ability to phosphorylate nucleolin. Infect Immun **2000**; 68:3121–8.
12. Ghigo E, Capo C, Tung CH, Raoult D, Gorvel JP, Mege JL. *Coxiella burnetii* survival in THP-1 monocytes involves the impairment of phagosome maturation: IFN-gamma mediates its restoration and bacterial killing. J Immunol **2002**; 169:4488–95.
13. Sato K, Tomioka H. Antimicrobial activities of benzoxazinorifamycin (KRM-1648) and clarithromycin against *Mycobacterium avium*–intracellulare complex within murine peritoneal macrophages, human macrophage–like cells and human alveolar epithelial cells. J Antimicrob Chemother **1999**; 43:351–7.
14. Scorneaux B, Ouadrhiri Y, Anzalone G, Tulkens PM. Effect of recombinant human gamma interferon on intracellular activities of antibiotics against *Listeria monocytogenes* in the human macrophage cell line THP-1. Antimicrob Agents Chemother **1996**; 40:1225–30.
15. Sly LM, Hingley-Wilson SM, Reiner NE, McMaster WR. Survival of *Mycobacterium tuberculosis* in host macrophages involves resistance to apoptosis dependent upon induction of antiapoptotic Bcl-2 family member Mcl-1. J Immunol **2003**; 170:430–7.
16. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer **1980**; 26:171–6.
17. Mui AL, Wakao H, O'Farrell AM, Harada N, Miyajima A. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. EMBO J **1995**; 14: 1166–75.
18. Rosen RL, Winestock KD, Chen G, Liu X, Hennighausen L, Finbloom DS. Granulocyte-macrophage colony-stimulating factor preferentially

activates the 94-kD STAT5A and an 80-kD STAT5A isoform in human peripheral blood monocytes. Blood **1996**; 88:1206–14.
19. Carryn S, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. Comparative intracellular (THP-1 macrophage) and extracellular activities of beta-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. Antimicrob Agents Chemother **2002**; 46:2095–103.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **1951**; 193:265–75.
21. Yamamoto Y, Klein TW, Tomioka M, Friedman H. Differential effects of granulocyte/macrophage colony-stimulating factor (GM-CSF) in enhancing macrophage resistance to *Legionella pneumophila* vs. *Candida albicans.* Cell Immunol **1997**; 176:75–81.
22. Blauer F, Groscurth P, Schneemann M, Schoedon G, Schaffner A. Modulation of the antilisterial activity of human blood–derived macrophages by activating and deactivating cytokines. J Interferon Cytokine Res **1995**; 15:105–14.
23. Fleming SD, Campbell PA. Some macrophages kill *Listeria monocytogenes* while others do not. Immunol Rev **1997**; 158:69–77.
24. Denis M. Growth of *Listeria monocytogenes* in murine macrophages and its modulation by cytokines; activation of bactericidal activity by interleukin-4 and interleukin-6. Can J Microbiol **1991**; 37:253–7.
25. DiPersio J, Billing P, Kaufman S, Eghtesady P, Williams RE, Gasson JC. Characterization of the human granulocyte-macrophage colony-stimulating factor receptor. J Biol Chem **1988**; 263:1834–41.
26. Griffin JD, Cannistra SA, Sullivan R, Demetri GD, Ernst TJ, Kanakura Y. The biology of GM-CSF: regulation of production and interaction with its receptor. Int J Cell Cloning **1990**; 8(Suppl 1):35–44.
27. Tran HT, Metcalf D, Cheers C. Anti-bacterial activity of peritoneal cells from transgenic mice producing high levels of GM-CSF. Immunology **1990**; 71:377–82.
28. Leenen PJ, Canono BP, Drevets DA, Voerman JS, Campbell PA. TNF-alpha and IFN-gamma stimulate a macrophage precursor cell line to kill *Listeria monocytogenes* in a nitric oxide–independent manner. J Immunol **1994**; 153:5141–7.
29. Ogawa R, Pacelli R, Espey MG, et al. Comparison of control of *Listeria* by nitric oxide redox chemistry from murine macrophages and NO donors: insights into listeriocidal activity of oxidative and nitrosative stress. Free Radic Biol Med **2001**; 30:268–76.
30. Zhan Y, Basu S, Lieschke GJ, Grail D, Dunn AR, Cheers C. Functional deficiencies of peritoneal cells from gene-targeted mice lacking G-CSF or GM-CSF. J Leukoc Biol **1999**; 65:256–64.
31. Hasegawa S, Miura T, Sasaki S, Madarame H, Nakane A. Dysregulation of interleukin-10 and interleukin-12 are involved in the reduced host resistance to *Listeria monocytogenes* infection in alymphoplastic aly mutant mice. FEMS Immunol Med Microbiol **2002**; 32:111–7.
32. Miller MA, Skeen MJ, Lavine CL, Kirk ZH. IL-12–assisted immunization generates $CD4^+$ T cell–mediated immunity to *Listeria monocytogenes.* Cell Immunol **2003**; 222:1–14.
33. Kolb-Maurer A, Kammerer U, Maurer M, et al. Production of IL-12 and IL-18 in human dendritic cells upon infection by *Listeria monocytogenes.* FEMS Immunol Med Microbiol **2003**; 35:255–62.
34. Nomura T, Kawamura I, Tsuchiya K, et al. Essential role of interleukin-12 (IL-12) and IL-18 for gamma interferon production induced by listeriolysin O in mouse spleen cells. Infect Immun **2002**; 70:1049–55.
35. Yonekura K, Kawakita T, Saito Y, Suzuki A, Nomoto K. Augmentation of host resistance to *Listeria monocytogenes* infection by a traditional Chinese medicine, ren-shen-yang-rong-tang (Japanese name: ninjin-youei-to). Immunopharmacol Immunotoxicol **1992**; 14:165–90.
36. Nicola N. GM-CSF. In: Oppenheim JJ, Feldmann M, eds. Cytokine reference: a compendium of cytokines and other mediators of host defense. San Diego: Academic Press, **2000**:899–910.
37. LEUKINE (Sagramostim) prescribing information (package insert; REV 0230-03). Richmond, CA: Berlex Laboratories, **2002**.