Differential immunocompetence of macrophages derived using macrophage or granulocyte-macrophage colony-stimulating factor

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Abstract: Macrophages derived in vitro from bone marrow progenitors (bone marrow-derived macrophages, BMDMs) using either macrophage colony-stimulating factor (CSF-1) or granulocyte-macrophage colony-stimulating factor (GM-CSF) as the myelopoietic stimulus display differential functional, morphological, and mRNA phenotypes. The data presented here demonstrate further that CSF-1- and GM-CSF-derived BMDMs differ in immunologic capacity. GM-CSF-derived BMDMs, when compared to CSF-1-derived BMDMs, showed greater cytolytic activity against tumor necrosis factor α (TNF-α)-resistant, but not TNF-α-sensitive, tumor targets. In contrast, CSF-1-derived BMDMs produced nitrite in response to lipopolysaccharide (LPS) alone, whereas GM-CSF-derived BMDMs required interferon γ plus LPS treatment. The two BMDM populations also showed differential sensitivities to LPS for secretion of TNF-α and nitrite, but the maximal inducible amounts of these factors and prostaglandin E_2 were similar between the BMDM populations. Lastly, GM-CSF-derived but not CSF-1-derived BMDMs showed an L-arginine-dependent listeriacidal activity. These results show that the functional heterogeneity of CSF-1- and GM-CSF-derived macrophages is limited and appears to result largely from differences in the activational signals required by each BMDM population to elicit a given function. *J. Leukoc. Biol.* 51: 69-76; 1992.

Key Words: *macrophage immunocompetence • macrophage colony-stimulating factor • granulocyte-macrophage colony-stimulating factor*

INTRODUCTION

Hematopoiesis is a continuous and highly regulated process. The differentiation of myeloid lineage-restricted progenitor cells is initially controlled by interleukin 3(IL-3), which pushes the progenitors along the maturation pathway to a point where they can respond to specific lineage-restricted glycoproteins called colony-stimulating factors (CSFs) [10, 20]. The CSFs then determine the specific mature cell type produced.

Because a functional link is formed between hematopoietic progenitors and peripheral lymphocytes through the production of multiple cytokines [3], it becomes important to distinguish between normal or "steady-state" hematopoiesis occurring in the absence of an immunological stimulus and "induced" hematopoiesis associated with an inffammatory response. Production of the macrophage (Mϕ) lineage from bone marrow progenitors is normally controlled by CSF-1 [19], which is constitutively produced by many tissues [25]. In response to invasive stimuli, however, monocyte numbers increase dramatically, as do serum levels of CSF-1 [26, 29]. In addition, T cell-derived granulocyte/macrophage-CSF (GM-CSF) appears in the serum. Like CSF-1, GM-CSF directs the maturation of Mϕs but is also capable of producing granulocytes from bipotential progenitor cells [10].

Macrophages are responsible for numerous immunological functions and inflammatory processes (for review see ref. 1). It is apparent, however, that Mϕs isolated from different anatomical sites display a diversity of phenotypes and functional capacities [4, 8, 38]. Because Mϕ function is dictated, in large part, by signals received from the immediate microenvironment, tissue-specific Mϕ heterogeneity results from the complement of factors present in a given tissue. It is also apparent, however, that functional heterogeneity exists within a given Mϕ preparation [6, 21]. Thus, Sorg [35] proposed that Mϕ differentiation is accompanied by the transient expression of certain phenotypes, and functional heterogeneity therefore results from the spectrum of maturational states within a given isolate. In support of this hypothesis, it was observed in this laboratory that the expression of the membrane form of IL-1 by bone marrow-derived macrophages (BMDMs) required at least 7 days of differentiation, whereas the secreted form of IL-1 appeared as early as day 3 of culture [28]. Major histocompatibility complex class (MHC) II gene expression was also maximal at 7 days of culture [31], and mRNA levels encoding the protooncogenes c-*myc* and c-*fms* varied as a function of the culture period [28].

Recent evidence also suggests a role for the hematopoietic growth factor encountered by progenitor cells in determining Mϕ functional capacity. Witsell and Schook [39] observed differential mRNA phenotypes for CSF-1- and GM-CSF-derived Mϕ colonies. Vogel and colleagues [12, 14, 15] observed CSF-1-derived BMDMs to have a greater phagocytic index but lower basal cytotoxicity and antigen-presenting capacity than GM-CSF-derived BMDMs. Further, CSF-1-derived Mϕs are more resistant to infection by vesicular stomatitis virus because of their enhanced production of interferon (IFN)-α/β [13]. The studies presented here further examine the functional specialization of Mϕ popula-

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Abbreviations: BMDM, bone marrow-derived macrophage; CFU, colony-forming unit; CPSR, controlled-processed serum replacement factor; CSF, colony-stimulating factor; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBSS, Hanks' balanced salt solution; IFN-γ, interferon γ; IL-3, interleukin-3; LCCM, L929 cell-conditioned medium; LPS, lipopolysaccharide; MHC, major histocompatibility complex; Mϕ macrophage; NK cells, natural killer cells; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor α.

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tions derived using different hematopoietic stimuli. We show differential tumoricidal and listeriacidal activities, nitrite secretion, and sensitivities to lipopolysaccharide (LPS) by CSF-1- and GM-CSF-derived BMDMs. The data suggest that Mϕ function following immune challenge can be "directed" by the recruitment of functionally distinct Mϕ populations via Mϕ maturation using CSF-1 or GM-CSF.

MATERIALS AND METHODS

Mice

Experiments were conducted using 8- to 12-week-old female, specific pathogen-free C3HeN mice obtained from Harlan Sprague Dawley (Indianapolis, IN). Mice were maintained in an environment-controlled chamber in groups of 5 for at least 1 week prior to use to allow recuperation following transport. Diets consisted of autoclaved commercial mouse chow and sterilized tap water.

Preparation of BMDM

Mice were sacrificed by cervical dislocation, and the femurs were removed and cleansed of tissue. Marrows were flushed from the femurs using Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS), disrupted by passage through a 23-gauge needle, collected by centrifugation, washed with 10 ml of HBSS, and cultured at a density of 10^6 cells/ml in polystyrene tissue culture dishes in 20 ml of DMEM supplemented with 10% horse serum, 10% controlled-processed serum replacement factor 1 (CPSR-1; Sigma Chemical Co., St. Louis, MO), vitamins, L-glutamine, and either 10% L929 cell-conditioned medium (LCCM) as a source of CSF-1 [29] or 300 U/ml recombinant murine GM-CSF (a gift from Steven Gillis, Immunex Corp., Seattle, WA). Following 6 days of incubation at 37°C in 7.5% CO_2, nonadherent cells were decanted, the adherent monolayer was washed twice with cold HBSS, and the cells were harvested by gentle scraping with a rubber policeman. Viable cell counts were determined by trypan blue dye exclusion, and the BMDMs were resuspended in DMEM suplemented with 10% fetal bovine serum (FBS). Identity as Mϕs was determined by nonspecific esterase staining using a commercial esterase staining kit (Sigma Chemical Co.) and a commercial Wright stain (Hema-3; Curtin-Matheson Scientific Inc., Houston, TX).

Preparation of LCCM

The murine L929 fibroblast cell line was used as a source of CSF-1 [19]. The cells were grown in 75-ml culture flasks in DMEM, 10% CPSR-2 to confluence, and the medium was replaced with 20 ml of fresh medium. After 5 days the medium was poured off, filtered through a 0.22-μm filter (Millipore Co., Bedford, MA), and stored at −20°C until used.

BMDM-Conditioned Medium

Precise densities of BMDMs (5×10^5) were placed in 24-well tissue culture plates in 1 ml of DMEM, 10% CPSR-1. Because attachment to plastic surfaces can induce transient expression of certain genes, including tumor necrosis factor α (TNF-α) [11], the BMDMs were incubated for 8 h and washed with HBSS prior to use. Fresh DMEM, 10% CPSR-1 was added along with the biological response modifiers to yield a final volume of 1 ml. At the indicated time, 900 μl of the supernatants was removed into sterile microfuge tubes, spun for 2 min, transferred to fresh microfuge tubes, and frozen at −80°C until use. When the BMDMs were to be primed, IFN-γ (10 U/ml) was added 4 h prior to the addition of endotoxin. Where indicated, prostaglandin E_2 (PGE_2) was added 2 h prior to endotoxin. Endotoxin (*Escherichia coli* O55:B5) was purchased from Difco Laboratories, Detroit, MI. Recombinant murine IFN-γ was a gift from the Schering Corporation through the American Cancer Society, and indomethacin, PGE_2, and actinomycin D were obtained from Sigma Chemical Co.

Measurement of TNF-α Activity

BMDM culture supernatants were collected 6 h following endotoxin treatment and examined for TNF-α against the TNF-α-sensitive, natural killer (NK) cell-resistant WEHI 164 cell line. Serial twofold dilutions were made in 96-well microtiter plates using RPMI 1640 without FBS in a total volume of 50 μl. Targets (4×10^5) were added in 50 μl RPMI 1640, 10% FBS containing 1 μg/ml actinomycin D. Plates were incubated for 18 h at 37°C in 7.5% CO_2. Cytotoxicity was determined by adding 20 μl of a 2.5 mg/ml saline solution of 3-(4,5-dimethylthiazol-2-yl) 3,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.). Following an additional 4-h incubation, 100 μl of the medium was removed, the MTT crystals were dissolved by addition of 100 μl of isopropanol, 0.04 N HCl, and absorbance was measured using a Biotek EL310 microplate reader at 550 nm with a reference wavelength of 690 nm. Maximal cytotoxicity was determined by incubating WEHI 164 targets in 0.01% Triton X-100, and 0% cytotoxicity was determined from targets incubated in medium without growth factors. Cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{OD_{\text{medium control}} - OD_{\text{sample}}}{OD_{\text{medium control}} - OD_{\text{Triton X-100 control}}} \times 100\%$$

TNF-α titers were determined by plotting sample dilution versus log % cytotoxicity. One unit of TNF-α activity was defined as the reciprocal of the dilution required to give 50% cytotoxicity. Pretreatment of samples with 4 U/ml neutralizing antibody against murine TNF-α (Endogen, Boston, MA), but not catalase (1000 U/ml) or superoxide dismutase (2000 U/ml), completely abolished their cytotoxic capacity against WEHI 164 targets (data not shown).

PGE_2 Measurement

BMDM supernatants were collected 24 h following the addition of endotoxin. Samples were diluted in HBSS and examined for the presence of PGE_2 using a commercial PGE_2 radioimmunoassay (New England Nuclear, Boston, MA).

Macrophage Cytotoxicity

BMDMs were replated into flat-bottom 96-well microtiter plates at a cell density of 2×10^5/well in 100 μl of DMEM and 10% FBS. After a 2-4-h adherence period, the cells were washed with HBSS, and triplicate wells were treated with 100 μl of the indicated agents for 18 h. Log phase cultures of the TNF-α-resistant K562 cell line were labeled with 200 μCi sodium [^{51}Cr]chromate (New England Nuclear) in 1 ml HBSS for 2 h. Alternatively, log phase L929 cells were in-

cubated overnight with 25 μCi [^{3}H]thymidine (New England Nuclear). Tumor targets were harvested, washed twice with DMEM, and allowed to incubate an additional 30 min in a large volume of medium. Following another wash, the cells were added to the BMDM at 2×10^4 cells/well (effector/target ratio of 10:1) in a final volume of 200 μl/well. After a 24-h incubation at 37°C in 7.5% CO_2, the plates were centrifuged at 200*g* for 5 min to pellet cell debris, and the top 100 μl of the medium was removed for anlaysis. Cytotoxicity was defined as

$$\% \text{ cytolysis} = \frac{\text{release from test sample} - \text{spontaneous release}}{\text{total count} - \text{spontaneous release}} \times 100$$

where maximal cytotoxicity was determined from wells treated with 2% sodium dodecyl sulfate (SDS), and spontaneous release was determined from wells consisting of targets with medium or experimental reagents.

Assay for NO_2^- Production

BMDM-conditioned medium (50 μl) was incubated in microtiter plates with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was measured using a microplate reader. The NO_2^- was determined using a sodium nitrite standard curve. All reagents were purchased from Sigma Chemical Co.

IL-6 Assay

The IL-6 activity in BMDM-conditioned medium was detected by measuring [^{3}H]Tdr uptake by the IL-6-dependent B9 cell line [34] (obtained with permission from L. A. Aarden through D. L. Laskin, Rutgers University). The assay was carried out in flat-bottom microtiter plates in a total volume of 200 μl. Samples were serially diluted using B9 medium (RPMI 1640, 5% FBS, 5×10^{-5} M β-mercaptoethanol), and 5000 B9 cells were added. Following a 72-h incubation, 2.5 μCi [^{3}H]Tdr was added per well and incubation continued for 18 h. Cells were collected using an automated cell harvester, and radioactivity was determined using liquid scintillation counting. The activity of IL-6 in the test supernatants was calculated on the basis of growth obtained in the presence of a rIL-6 (Amgen, Thousand Oak, CA) standard titration curve where 1 U was defined as causing half-maximal B9 cell proliferation.

In Vitro Microbicidal Activity

In vitro killing of *Listeria monocytogenes* was monitored by the addition of 10^5–10^6 viable bacteria to 10^5 freshly isolated BMDMs in 0.2 ml in flat-bottom microtiter plates and spinning at 450*g* for 2 min to achieve contact between the bacteria and the BMDMs. Following a 20-min incubation, nonadherent bacteria were washed away and the BMDMs in half of the wells were lysed by the addition of cold 0.01% SDS (100 μl) under visual control with a microscope. The resulting bacterial suspensions were rapidly diluted in saline (0.9%). DMEM (25 μl) with or without response modifiers was added to the remaining wells, and plates were incubated an additional 1 h at 37°C and then lysed by the addition of 75 μl of 0.1% SDS to release intracellular bacteria. Aliquots (10 μl) of all lystates were diluted, and the number of viable bacteria was determined by plate counting on trypticase soy agar. The ratio of colony-forming units (CFU) after the 20-min and 1-h incubations indicated the ability of BMDM to kill *Listeria*.

RESULTS

Cell-Mediated Tumoricidal Activity

Mϕs utilize several mechanisms for killing tumor cells, and the lethal hit phase of the cytotoxic reaction depends on the specific sensitivity of the tumor cell in question [18, 23]. We examined the ability of BMDMs to kill TNF-α-sensitive L929 and TNF-α-resistant K562 tumor cells (Table 1). The BMDM populations showed similar abilities to cytolyze L929 targets and required the classical IFN-γ plus LPS, priming, and triggering treatment protocol to become fully tumoricidal. Against K562 targets, GM-CSF-derived BMDMs showed higher basal tumoricidal activity than CSF-1-derived BMDMs and required only LPS to become maximally cytolytic. In contrast, CSF-1-derived BMDMs required IFN-γ plus LPS treatment for maximal killing activity. These results suggested that CSF-1- and GM-CSF-derived BMDMs differed in their capacity for one or more tumoricidal mechanisms.

TNF-α Production

Although no difference was observed between the BMDM populations in their ability to kill L929 targets, TNF-α-dependent, direct Mϕ-mediated tumor killing is a function independent of TNF-α secretion [23]. In addition, the TNF-α secretion capacity varies between Mϕ isolates [5, 8]. Table 2 shows that conditioned medium from untreated BMDM cultures contained no TNF-α activity. CSF-1-derived BMDMs produced 2- to 3-fold more TNF-α at low LPS concentrations than GM-CSF-derived BMDMs but only 1.3-fold more TNF-α at high LPS concentrations. IFN-γ alone did not induce TNF-α secretion, but treating the BMDMs with IFN-γ for 4 h prior to the addition of LPS significantly aug-

TABLE 1. Tumoricidal Activity of CSF-1- and GM-CSF-Derived BMDMs Against TNF-α-Sensitive and -Resistant Targets[a]

	L929 targets		K562 targets	
Treatment	CSF-1 BMDMs	GM-CSF BMDMs	CSF-1 BMDMs	GM-CSF BMDMs
Medium	24.7 ± 1.4	23.5 ± 1.1	5.3 ± 1.0	19.7 ± 0.4
IFN-γ (10 U/ml)	30.0 ± 0.8	25.0 ± 0.4	9.6 ± 0.8	22.3 ± 2.5
LPS (0.5 μg/ml)	27.6 ± 2.1	24.0 ± 5.3	15.6 ± 0.8	34.9 ± 2.0
IFN-γ + LPS	76.3 ± 4.8	82.7 ± 4.7	21.6 ± 2.1	30.0 ± 2.2

[a]Values given are % specific cytotoxicity ± standard error for triplicate wells for one of two representative experiments. Tumoricidal activity was assessed as described in Materials and Methods against ^{3}H-labeled L929 or ^{51}Cr-labeled K562 cells.

TABLE 2. TNF-α Production by CSF-1- and GM-CSF-Derived BMDMs[a]

Treatment		TNF-α (U/5 × 10^5 BMDMs)	
IFN-γ (U/ml)	LPS (ng/ml)	CSF-1 BMDMs	GM-CSF BMDMs
–	–	< 5	< 5
	0.5	< 25	< 25
	5	297 ± 80	< 50
	50	276 ± 128	87 ± 37
	500	477 ± 170	356 ± 145
10	–	< 10	< 10
	0.5	< 100	< 100
	5	474 ± 347	221 ± 148
	50	900 ± 232	374 ± 38
	500	1660 ± 38	1187 ± 200
10 + actinomycin D[b]	500	< 10	< 10

[a] BMDM (5 × 10^5) were placed in 24-well plates for 8 h as described in Materials and Methods. The cells were washed, and 0.5 ml of medium or medium containing 10 U/ml IFN-γ was added. Following an additional 4-h incubation, 0.5 ml of medium or medium containing LPS was added, and conditioned medium was collected after 6 h and stored at −80°C until assayed. Values are given as mean ± standard error for two or three samples for one of two representative experiments.

[b] Actinomycin D was added to 1 μg/ml 30 min prior to the addition of LPS.

mented the TNF-α production of both populations. However, the CSF-1-derived BMDM population remained more sensitive to low concentrations of LPS. Conditioned medium from BMDMs treated with actinomycin D contained no TNF-α activity, indicating that neither BMDM population retained intracellular pools of TNF-α.

Nitrite Secretion

Hibbs and co-workers have demonstrated the participation of nitric oxide in the L-arginine-dependent killing of both tumor and microbial targets [16, 37]. In the present study, conditioned medium from BMDMs was obtained following a 24-h stimulation with LPS and examined for nitrite content as a measure of total nitric oxide production (Table 3). Unstimulated BMDMs accumulated no nitrite, and IFN-γ did not induce nitric oxide production. CSF-1- but not GM-CSF-derived BMDMs produced nitrite in response to high concentrations of LPS alone. As observed for TNF-α production, a 4-h treatment with IFN-γ prior to the addition of LPS greatly augmented nitrite accumulation in the medium. More importantly, GM-CSF-derived BMDMs produced as much nitrite as CSF-1-derived BMDMs. As expected, depleting L-arginine from the medium or inhibiting protein synthesis with actinomycin D (data not shown) abrogated nitrite production. Thus, GM-CSF-derived BMDMs require prior treatment with IFN-γ in order to respond to LPS for nitric oxide production. Lastly, nitrite accumulation in the medium did not occur until after 6 h of stimulation (Table 4), consistent with an autocrine mechanism for nitrite production by TNF-α [22].

Secretion of PGE_2

PGE_2 mediates numerous inflammatory processes, including the autoregulatory control of TNF-α production by Mϕs, and is immunosuppressive [24]. Because Mϕs represent a major cell type producing PGE_2 in inflammatory lesions [33], we compared CSF-1- and GM-CSF-derived BMDMs for PGE_2 production. Both populations constitutively secreted very low levels of PGE_2 (Table 5) and responded to LPS in a dose-dependent manner. IFN-γ dramatically augmented PGE_2 production at low LPS concentrations. The BMDM populations differed only at very low LPS concentrations. As expected, indomethacin completely inhibited PGE_2 production. Table 6 shows a biphasic PGE_2 response to LPS. About 30% of the total PGE_2 accumulating by 24 h appeared rapidly in the medium, but most of the PGE_2 secretion did not occur until after 6 h. However, TNF-α production was essentially complete by 6 h because TNF-α did not continue to accumulate in the medium after this time and replacing the medium after 6 h with fresh medium resulted in no further TNF-α secretion. In contrast, the amount of PGE_2 accumulating in fresh medium between 6 and 24 h nearly equaled the difference between the 6- and 24-h time points. Taken together, Tables 4 and 6 show that CSF-1- and GM-CSF-derived BMDMs do not differ kinetically in their response to LPS for TNF-α, nitrite, and PGE_2 production.

IL-6 Production

The recently defined cytokine IL-6 is now known to have a wide range of immunological effects, and Mϕs are documented sources of IL-6 [34]. The IL-6-dependent B9 cell line was used to detect IL-6 in 24-h conditioned medium from BMDMs (Table 7). Constitutive IL-6 production was not observed for either population, and the BMDM popula-

TABLE 3. Accumulation of Nitrite in Conditioned Medium from CSF-1- and GM-CSF-Derived BMDM[a]

		Nitrite (nmol/5 × 10^5 BMDMs/24 h)	
IFN-γ (U/ml)	LPS (μg/ml)	CSF-1 BMDMs	GM-CSF BMDMs
–	–	−4.1 ± 1.1	−4.3 ± 0.8
	0.5	1.4 ± 0.8	1.1 ± 0.5
	5	3.3 ± 2.8	0.7 ± 1.0
	50	7.9 ± 0.8	2.9 ± 1.4
	500	22.1 ± 1.1	9.2 ± 0.6
10	–	−2.6 ± 0.6	−1.7 ± 1.1
	0.5	12.2 ± 1.3	8.3 ± 2.1
	5	24.7 ± 1.8	33.8 ± 3.1
	50	21.1 ± 2.5	37.6 ± 0.5
	500	36.4 ± 0.7	33.3 ± 6.9
10 without L-arginine[b]	500	6.5 ± 0.6	8.9 ± 0.1

[a] BMDMs were prepared and treated as described in footnote a of Table 2. Conditioned medium was collected at 24 h and assessed for nitrite content as described in Materials and Methods. Values given are the mean ± standard error for two or three samples for one of two representative experiments.

[b] Following overnight incubation of the BMDMs the monolayers were washed, and L-arginine-free medium was added at the time of stimulation.

TABLE 4. Kinetics of Nitrite Accumulation in BMDM-Conditioned Medium

Treatment	Time (h)	Nitrite (nmol/5 × 10^5 BMDMs) CSF-1 BMDMs	GM-CSF BMDMs
None	24	- 5.5	- 5.7
IFN-γ (10 U/ml)	24	- 2.6	- 1.7
IFN-γ (10 U/ml)	6	- 5.2 ± 0.0	- 3.8 ± 0.6
+	12	9.3 ± 1.5	12.7 ± 1.6
LPS (0.5 μg/ml)	24	27.6 ± 0.6	34.6 ± 6.0

[a] Values for untreated and IFN-γ-treated samples were obtained from single wells. All other values are given as the mean ± standard error for two or three samples for one of two representative experiments.

TABLE 5. LPS Induction of PGE_2 Secretion by CSF-1- and GM-CSF-Derived BMDMs[a]

IFN-γ (U/ml)	LPS (ng/ml)	PGE_2 (nmol/5 × 10^5 BMDMs/24 h) CSF-1 BMDMs	GM-CSF BMDMs
–	–	0.6 ± 0.1	1.2 ± 0.1
	0.5	0.9 ± 0.1	2.2 ± 0.4
	5	18.7 ± 1.7	10.5 ± 5.3
	50	23.2 ± 2.8	29.5 ± 1.5
	500	32.0 ± 2.4	36.9 ± 2.1
10	–	0.5 ± 0.1	0.8 ± 0.1
	0.5	16.1 ± 2.2	3.9 ± 0.4
	5	41.1 ± 2.5	45.8 ± 2.8
	50	38.5 ± 4.5	44.6 ± 0.5
	500	49.5 ± 1.5	46.7 ± 1.9
10 + indomethacin[b]	500	0.6 ± 0.2	0.6 ± 0.1

[a] BMDMs were prepared and treated as described in footnote a of Table 2 and conditioned medium was assessed at 24 h for PGE_2 content as described in Materials and Methods. Values represent the mean ± standard error for two or three samples for one of two representative experiments.
[b] Indomethacin (5×10^{-7}M) was added at the same time as the LPS.

tions produced similar amounts of IL-6 in response to LPS. IFN-γ did not significantly enhance IL-6 secretion by either BMDM population.

Killing of Intracellular *Listeria*

Listeria monocytogenes is a facultative intracellular pathogen of Mϕs. The course and outcome of listerial infection depend largely on the activational status of the Mϕs, but T cells are required for complete resolution of infection [30, 33]. To determine whether Mϕs derived by GM-CSF afford greater bacteriacidal activity than CSF-1-derived Mϕs, in vitro killing of intracellular *Listeria* was measured (Table 8). To account for possible differences in phagocytic capacities of CSF-1- and GM-CSF-derived BMDMs, the number of bacteria in Mϕs following a 20-min exposure to *Listeria* was used as a rough estimate of phagocytosis. Untreated GM-CSF-derived BMDMs showed greater phagocytosis than untreated CSF-1-derived BMDMs, but the reverse was true if the BMDMs were stimulated with IFN-γ and/or LPS prior to the addition of *Listeria*. In particular, IFN-γ plus LPS-stimulated GM-CSF-derived BMDMs showed a significant decrease in CFU/well.

Killing of intracellular *Listeria* was calculated by determining the number of viable *Listeria* remaining within the BMDMs following an additional 60-min incubation and dividing by the CFU of *Listeria* present in that population following the 20-min phagocytosis period. Untreated GM-CSF-derived BMDMs had greater listeriacidal activity than untreated CSF-1-derived BMDMs. IFN-γ alone did not enhance killing by either population, but LPS alone significantly inhibited listeriacidal activity by both BMDM populations. Interestingly, IFN-γ plus LPS treatment induced high levels of killing by GM-CSF- but not CSF-1-derived BMDMs. The addition of superoxide dismutase (10^4 U/ml) at the time of *Listeria* exposure did not promote *Listeria* survival in IFN-γ plus LPS-stimulated BMDMs (data not shown).

Because recent evidence suggests a role for L-arginine-dependent killing mechanisms for intracellular parasites [16], killing of intracellular *Listeria* in the absence of L-arginine was determined (Table 9). Depletion of arginine from the medium did not significantly alter the phagocytosis of *Listeria* (data not shown), nor did it inhibit the basal listeriacidal activities of the BMDM populations. However, IFN-γ plus LPS-induced killing by GM-CSF-derived BMDMs was completely abrogated by arginine-free conditions. Thus, in constrast to CSF-1-derived BMDMs, GM-CSF-derived BMDMs are induced by IFN-γ plus LPS to become listeriacidal, and this killing mechanism requires the participation of L-arginine-derived molecules, presumably nitric oxide.

DISCUSSION

Although the hematopoietic capacity of GM-CSF has been well characterized, determination of the immunocompetence of GM-CSF-derived Mϕs has lagged. Vogel and colleagues [12-15] have shown CSF-1- and GM-CSF-derived BMDMs to be morphologically and functionally distinct, and our own

TABLE 6. The Kinetics of LPS-Induced PGE_2 and TNF-α Secretion by BMDMs[a]

Time (h)	TNF-α (U/ml) CSF-1 BMDMs	GM-CSF BMDMs	PGE_2 (ng/5 × 10^5 BMDMs) CSF-1 BMDMs	GM-CSF BMDMs
0	<2	<2		
1	<5	34		
2	1230	799	11.1 ± 0.5	11.8 ± 0.8
6	981	967	12.0 ± 0.3	16.8 ± 0.4
24	579	892	33.0 ± 0.6	41.7 ± 5.2
6→ 24[b]	<20	<20	17.1 ± 1.7	19.4 ± 10.9

[a] BMDMs were treated with IFN-γ (10 U/ml) and LPS (0.5 μg/ml) as described in the footnotes to Table 2. Values for TNF-α were obtained from single wells, whereas value for PGE_2 are the mean ± SE for two identically treated wells.
[b] BMDMs were treated with IFN-γ plus LPS for 6 h, washed, and fresh medium without LPS was added. Following an additional 18-h incubation, conditioned medium was collected for anlaysis as described in Materials and Methods.

TABLE 7. IL-6 Production by CSF-1- and GM-CSF-Derived BMDM[a]

Treatment	IL-6 (U/5 × 10^5 BMDMs/24 hr)	
	CSF-1 BMDMs	GM-CSF BMDMs
None	0.5 ± 0.7	0.3 ± 1.1
LPS (0.5 μg/ml)	30.5 ± 1.9	28.0 ± 2.1
IFN-γ (10 U/ml) + LPS	37.9 ± 3.9	30.3 ± 5.8

[a]BMDMs were prepared and treated as described in the footnotes to Table 2. Conditioned medium from 24-h cultures was collected and assessed for IL-6 activity as described in Materials and Methods. Values are given as the mean ± SE for two or three samples for one of two representative experiments.

laboratory reported unique mRNA phenotypes for these BMDM populations [39]. The data presented here extend previous observations by showing differential tumoricidal activity, signal requirements for nitrite secretion, and listeriacidal capacity of CSF-1- and GM-CSF-derived BMDMs. These findings further suggest that Mϕs derived from progenitor cells under the influence of different colony-stimulating factors contribute to the functional heterogeneity of peripheral Mϕ populations. A similar situation exists for mast cells in that IL-4 produces connective-tissue mast cells, whereas IL-3 supports the proliferation of mucosal mast cells [20].

The functional differences between CSF-1- and GM-CSF-derived BMDMs are, however, limited. We observed no significant differences between the BMDM populations for potential production of TNF-α, nitrite, PGE_2, or IL-6. Furthermore, the kinetics of TNF-α, nitrite, and PGE_2 secretion were the same for the two BMDM populatons and are consistent with reports for other Mϕ preparations]2, 5, 16]. Instead, it was observed that the BMDM populations often displayed differential sensitivities to LPS or required additional stimulation to express a given function. GM-CSF-derived BMDMs required only LPS for effective killing of K562 cells but produced little nitrite in response to LPS unless first treated wtih IFN-γ. Conversely, CSF-1-derived BMDMs required the classical IFN-γ plus LPS treatment protocol to become tumoricidal for K562 cells but secreted nitrite in response to high concentrations of LPS alone. In addition, the susceptibility of GM-CSF-derived BMDMs to the cytopathic effects of vesicular stomatitis virus could be abrogated by the addition of IFN-α/β in concentrations equivalent to those found in CSF-1 cultures [13]. Therefore, much of the heterogeneity observed between CSF-1- and GM-CSF-derived BMDMs originates not with the differential ability to perform a specific function but with the signals required for expression of that activity. These data also confirm independent regulation for the cytolytic mechanisms used by Mϕs [18] and show CSF-1- and GM-CSF-derived BMDMs to differentially regulate these mechanisms.

Falk et al. [14] observed GM-CSF-derived BMDMs to have greater basal and induced tumoricidal activity against TNF-α-resistant P815 tumor targets. The present study also showed greater tumoricidal activity by GM-CSF-derived BMDMs against TNF-α-resistant targets, but in constrast to the previous report, tumor killing was induced by LPS treatment alone. However, Falk et al. used LPS-hyporesponsive (LPS^d) C3H/HeJ mice, which require IFN-γ treatment to become LPS responsive. Falk and Vogel [12] also observed CSF-1-derived BMDMs to have a greater phagocytic capacity than GM-CSF-derived BMDMS, but we observed that unstimulated GM-CSF-derived BMDMs ingested more *Listeria* than CSF-1-derived BMDMs. The reason for these conflicting results is unclear at present, but may reflect a difference in the mechanism of *Listeria* uptake compared to latex ingestion or Fc receptor-mediated phagocytosis. Further, only viable *Listeria* were quantitated and this may not necessarily reflect total listerial uptake. Following activation with IFN-γ plus LPS, CSF-1-derived BMDMs did contain more *Listeria* than GM-CSF-derived BMDMs.

GM-CSF-derived BMDMs, but not CSF-1-derived BMDMs, could be made highly listeriacidal by IFN-γ plus LPS. The mechanism by which this bacteriacidal activity is mediated is unknown but is different from the constitutively expressed mechanism in that killing by activated GM-CSF-derived BMDMs requires the presence of L-arginine. Reactive oxygen products probably do not participate in the listeriacidal function of activated BMDMs because superoxide dismutase did not increase *Listeria* CFU in BMDM lysates and LPS treatment likely abolished superoxide production by the BMDMs [32]. The L-arginine-derived product is probably nitric oxide because GM-CSF-derived BMDMs neither secrete nitrite nor kill *Listeria* following LPS treatment alone. Nitric oxide has been shown to play a role in the killing of tumors [37], protozoan parasites [16], fungi [2], and gram-negative intracellular pathogens [7], and this is the first report to demonstrate L-arginine-dependent killing of gram-positive bacterial pathogens.[1] It must be noted, however, that nitric oxide itself is incapable of killing *Listeria* because CSF-1-derived BMDMs are potent sources of nitrite, yet are not activated for *Listeria* killing by IFN-γ plus LPS.

The differential function of CSF-1- and GM-CSF-derived BMDMs results from the hematopoietic and not the activational properties of the CSF. Progenitor cells received

[1]Following submission of this report, Denis showed reactive nitrogen intermediate-dependent killing of *Mycobacterium avium* [9].

TABLE 8. Killing of Intracellular *Listeria* by CSF-1- and GM-CSF-Derived BMDMs[a]

Treatment	CSF-1 BMDMS			GM-CSF BMDMs		
	CFU after phagocytosis (× 10^3)	CFU after culture (× 10^3)	% killing	CFU after phagocytosis (× 10^3)	CFU after culture (× 10^3)	% killing
Medium	8.7 ± 0.1	4.9 ± 1.3	43.7 ± 10.3	13.2 ± 2.5	4.6 ± 0.9	65.2 ± 4.8
IFN-γ (10 U/ml)	14.9 ± 1.2	8.4 ± 0.9	43.6 ± 4.5	11.9 ± 1.1	6.0 ± 0.9	49.4 ± 2.6
LPS (0.5 μg/ml)	14.2 ± 0.4	10.2 ± 1.9	28.2 ± 9.4	12.2 ± 0.5	9.0 ± 1.3	26.2 ± 7.4
IFN-γ + LPS	15.1 ± 0.2	11.4 ± 1.5	24.5 ± 6.9	4.8 ± 1.0	0.9 ± 0.1	80.5 ± 1.8

[a]BMDMs (10^5) were prepared and treated as described in the footnotes to Table 2 in microtiter plates. BMDMs were incubated with 5 × 10^5 *Listeria* for 20 min, at which time all wells were washed and half of the wells were lysed by addition of cold 0.01% SDS. Aliquots were removed and diluted for determination of CFU. The remaining wells received fresh medium and were incubated an additional 60 min, at which time the BMDMs were lysed. Aliquots were removed for determination of remaining viable *Listeria*. Values represent the mean ± SE for two or three wells for one of three representative experiments.

TABLE 9. Inhibition of Induced Listeriacidal Activity by Arginine-Free Medium[a]

Treatment	% killing of intracellular *Listeria*	
	CSF-1 BMDMs	GM-CSF BMDMs
None	43.7 ± 10.3	65.2 ± 4.4
Without L-arginine	44.6 ± 2.2	71.9 ± 4.1
IFN-γ + LPS	24.5 ± 7.1	81.3 ± 1.7
IFN-γ + LPS without L-arginine	14.9 ± 3.6	12.2 ± 4.1

[a]BMDMs were prepared as described in the footnote to Table 8. Following incubation overnight, the medium was replaced with fresh medium with or without L-arginine, with or without IFN-γ (10 U/ml). After 4 h, LPS was added in medium with or without arginine to 0.5 μg/ml, and the BMDMs were incubated 2 h. *Listeria* (5×10^5 CFU) was added, and the percent killing was determined as above. Values represent the mean ± SE for two or three wells for one of two representative experiments.

only a single pulse of CSF at the time of plating, and all BMDMs were washed extensively, incubated overnight, and washed again to ensure removal of residual CSF prior to functional analysis. Furthermore, the functional phenotype of the BMDMs does not correspond to published reports or our own observations (manuscript in preparation) concerning the biological response modifier properties of the CSFs. For example, treatment of Mϕs with GM-CSF for more than 12 h inhibits TNF-α synthesis and renders the Mϕs refractive to LPS [17], yet GM-CSF-derived BMDMs responded to LPS for TNF-α production and all other functions examined.

Progenitor cells themselves are heterogeneous in size and proliferative capacity, even with colonies that yield only one morphological type of cell [26]. The experiments described here examine functional phenotypes of BMDM populations and, therefore, do not distinguish functional heterogeneity within individual clones. A limited number of phenotypes exist for differentiating Mϕ colonies [39], and the proportion of these phenotypes changes as a function of both the culture period and hematopoietic stimulus. Thus, Mϕ functional heterogeneity results from a combination of hematopoietic stimulus, maturation stages, and variational responses of progenitor cells.

A potential role for secondary mediators produced in response to CSFs as part of a cytokine cascade leading to Mϕ maturation must also be considered in the differentiation of functionally heterogeneous BMDMs by CSF-1 and GM-CSF. It has been shown that CSF-1 induces the synthesis of IFN-α/β during BMDM expansion, and this endogenous product can be used as an autocrine signal for Mϕ differentiation [27]. GM-CSF bone marrow cultures contain little or no IFN-α/β [13], and it is therefore suggested that this intrinsic difference accounts for many of the functional differences between CSF-1- and GM-CSF-derived BMDM populations. It can also not be ruled out that GM-CSF itself causes the production of other secondary mediators of Mϕ differentiation. It remains to be tested whether the addition of IFN-α/β will confer the complete CSF-1-derived functional phenotype upon GM-CSF-derived BMDMs. In any event, the net effect in vivo would be the same following immune challenge; the release of GM-CSF and the differentiation of an Mϕ population that is functionally distinct from constitutively produced, CSF-1-derived Mϕs. Thus, the recruitment of Mϕs derived under the influence of GM-CSF contributes to the heterogeneity of peripheral Mϕ populations and likely is of physiologic importance in the remediation of immune insult.

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