Role of Granulocyte/Macrophage Colony-Stimulating Factor in Zymocel-Induced Hepatic Granuloma Formation

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To examine the role of granulocyte/macrophage colony-stimulating factor (GM-CSF) in inflammatory granuloma formation, we injected GM-CSF-deficient (GM-CSF^{-/-}) mice and wild-type (GM-CSF^{+/+}) mice intravenously with 2 mg of zymocel, and mice were killed at various intervals for examination. In GM-CSF^{-/-} mice, we demonstrated a marked delay of zymocel-induced hepatic granuloma formation until 5 days after zymocel injection with a rapid reduction in numbers of granulomas at 10 days until their disappearance. In the early phase of granuloma formation, monocyte infiltration and differentiation of monocytes into macrophages were impaired in GM-CSF^{-/-} mice compared with GM-CSF^{+/+} mice. The percentages of [³H]thymidine-labeled macrophages at 2 days after zymocel injection were lower in the GM-CSF^{-/-} mice than in the GM-CSF^{+/+} mice. The DNA nick-end-labeling method demonstrated increased numbers of apoptotic cells in and around hepatic granulomas of GM-CSF^{-/-} mice from 8 days after zymocel injection, and electron microscopy detected apoptotic bodies. Granuloma macrophage digestion of glucan particles and activation of macrophages were similar in the two types of mice. *In situ* hybridization demonstrated expression of GM-CSF mRNA in the endothelial cells, hepatocytes, and some granuloma cells in the GM-CSF^{+/+} mice but not in the GM-CSF^{-/-} mice. These results provide evidence that GM-CSF is important for the influx of monocytes into hepatic granulomas, for differentiation of monocytes into macrophages, and for proliferation and survival of macrophages within hepatic granulomas. (*Am J Pathol* 2001, 158:131-145)

In humans and animals, the liver is the most important organ for defense against pathogenic microorganisms invading through the portal vein and hepatic arteries.

Kupffer cells are involved in the removal of a variety of macromolecular substances. However, when these substances cannot be digested by Kupffer cells, granulomas are formed in the hepatic sinusoids because of proliferation of Kupffer cells, infiltration of monocytes, and differentiation of these monocytes into macrophages, accompanied by transformation of granuloma macrophages into epithelioid cells and multinuclear giant cells.¹ Hepatic granulomas can be produced experimentally by different substances, including β -glucan (zymosan or zymocel),²⁻⁷ eggs of *Schistosoma mansoni*,⁸ *Corynebacterium parvum*,^{9,11} *Mycobacterium bovis*, *Bacillus Calmette Guérin*,^{9,12} *Mycobacterium avium*,¹³ *Listeria monocytogenes*,¹⁴ and *Leishmania*.¹⁵ Among these substances, β -glucan, which is composed of β -1,3-polyglucose, provides the strongest stimulation of macrophages,¹⁶ neutrophils,¹⁷ and natural killer cells.¹⁸ Macrophages can take up β -glucan via a specific receptor,¹⁹ which has been recently clarified as complement receptor type 3 (CR3, or CD11b/CD18).²⁰ The processes of hepatic granuloma formation have been studied with β -glucan in different mouse models, such as mice depleted of Kupffer cells by administration of liposome-entrapped dichloromethylene diphosphonate,² mice with severe monocytopenia induced by administration of strontium-89 (⁸⁹Sr),^{4,5} osteopetrosis (op/op) mice defective in production of functional macrophage-colony stimulating factor (M-CSF) protein,³ interleukin-5 (IL-5) transgenic mice,²¹ mice deficient in class A type I and type II macrophage scavenger receptor (MSR-A),¹⁰ mice deficient in CC chemokine receptor 2 (CCR-2),²² and immunodeficient mice.^{6,7} In Kupffer cell-depleted mice, ⁸⁹Sr-induced monocytopenic mice, op/op mice, MSR-A-deficient mice, and nude mice or severe combined immunodeficient (scid) mice, hepatic granuloma formation is severely impaired, whereas it is accelerated in IL-5 transgenic mice and xid mice. However, the role of granulocyte/macrophage colony-stimulating factor (GM-CSF) in hepatic granuloma formation has not been clarified *in vivo*.

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GM-CSF is a 23-kd glycoprotein known as a hematopoietic growth factor required for the proliferation and survival of hematopoietic cells committed to granulocytic and macrophage cell lineages and myeloid leukemic cells,^{23–26} and for differentiation of these cells into neutrophilic or eosinophilic granulocytes, macrophages, bone marrow macrophages, or dendritic cells.^{26–30} GM-CSF increases the responsiveness of tissue macrophages to M-CSF.²⁶ In addition to stimulating the production of granulocytes, macrophages, and dendritic cells, GM-CSF has a pronounced capacity to increase the function of these cells in a variety of immune reactions.³¹ Its *in vivo* effects include rapid leukocytosis,³¹ increased numbers of granulocytes and macrophages in tissues,³¹ stimulation of phagocytosis and superoxide production by neutrophils and macrophages,³² induction of class II major histocompatibility complex expression and urokinase-type plasminogen activator production by monocyte/macrophages,³³ enhancement of granulocyte and monocyte cell adhesion,^{34,35} augmentation of antigen-presenting function of macrophages,³⁶ enhancement of production of cytokines by mononuclear cells,³⁷ and enhancement of chemotaxis of neutrophils, monocytes, or dendritic cells and of transendothelial migration of monocytes.^{38–40} GM-CSF is also important for survival of hemopoietic cells because the human GM-CSF mutant E21R selectively binds to the α chain of GM-CSF receptor, behaves as a GM-CSF antagonist, and causes apoptosis of normal and malignant hemopoietic cells.⁴¹ Studies of the signal transduction pathway of GM-CSF showed that stimulation by GM-CSF increases protein tyrosine phosphorylation through activation of multiple tyrosine kinases such as Src-like tyrosine kinases, Fes, and p97.^{42–44} GM-CSF transgenic mice develop a lethal disease accompanied by excessive accumulation of macrophages in the eyes, muscles, peritoneal and pleural cavities, liver, and lungs.^{45,46} In these transgenic mice, peritoneal macrophages demonstrated long survival via self-renewal independently of blood monocytes.^{45,46} Two groups of investigators independently generated GM-CSF-deficient (GM-CSF^{-/-}) mice and their studies reported the development of pulmonary alveolar proteinosis.^{47,48} However, these studies have also indicated that GM-CSF is not essential *in vivo* in GM-CSF-deficient mice for maintenance of myeloid progenitor cells at normal levels and that monocytes/macrophages and dendritic cells develop normally in these mice.^{47,48} GM-CSF is also synthesized under the influence of estrogenic hormonal stimulation by uterine luminal and glandular epithelial cells in humans and other animals,^{49–51} and it is important for intercellular communication in the female reproductive system and fertility of mice.⁵² In a study of collagen-induced arthritis in GM-CSF-deficient mice, Campbell and colleagues⁵³ demonstrated that inflammatory cell responses were significantly reduced in the mutant mice, suggesting that GM-CSF is required for the development of this type of arthritis. However, little is known about the role of GM-CSF in hepatic granuloma formation *in vivo*.

In this study, we injected zymocel (β -glucan) intravenously into the GM-CSF-deficient mice and wild-type

mice to examine how these mutant animals respond to this macrophage stimulant *in vivo*. After injection, the GM-CSF-deficient mice and wild-type mice were killed at various intervals, and granuloma formation in the liver of the animals was examined.

Materials and Methods

Animals

Mice deficient in GM-CSF production were kindly supplied by Dr. Glenn Dranoff and co-workers⁴⁷ and germ-line transmitters of a mutant GM-CSF allele were crossed with C57BL/6 mice to produce mice heterozygous for a disrupted GM-CSF gene (GM-CSF^{+/-}). The heterozygous (GM-CSF^{+/-}) mice were mated to yield the homozygous mutant (GM-CSF^{-/-}) mice, wild-type (GM-CSF^{+/+}) mice, and heterozygous (GM-CSF^{+/-}) mice. Animals were kept in the Animal Research Center at the Kumamoto University School of Medicine, were fed with a standard rodent chow and water *ad libitum*, and were housed with same sex littermates in sawdust-lined cages. For genotyping, tissues were taken from the tails of mice at 3 or 4 weeks after birth and polymerase chain reaction (PCR) was performed. Sequences of primers used are as follows: for normal sense: 5'-ACACAGAAGTTTG-GCTCTGG-3', for antisense: 5'-GGCAGTATGTCTGG-TAGTAG-3', and for disrupted GM-CSF gene antisense: 5'-GTGGATGTGGAATGTGTG CG-3' to distinguish GM-CSF^{-/-} mice from GM-CSF^{+/+} and GM-CSF^{+/-} mice. Homozygous and wild-type mice of both sexes were used at 8 to 20 weeks of age. Zymocel (2 mg) (Alphabeta Technology, Worcester, MA) was injected into the tail vein of GM-CSF^{-/-} and GM-CSF^{+/+} mice. All mice were killed under ether anesthesia at 2, 3, 5, 8, 10, 14, and 21 days after injection and their livers were removed. Some liver tissues were frozen in liquid nitrogen and stored for mRNA analysis or were embedded in OCT compound (Miles, Elkhart, IN) for immunohistochemistry; others were fixed in 10% formaldehyde solution for light microscopic studies. To examine the effects of GM-CSF, GM-CSF^{-/-} mice were injected daily with 5 ng of recombinant murine (rm) GM-CSF from 5 days before zymocel injection and throughout the experiment; because this dose is most effective for differentiation, proliferation, and survival of macrophages.³⁰

Light Microscopy

Liver tissues fixed in 10% formaldehyde were embedded in paraffin, paraffin sections were cut 3- μ m thick, and slides were stained with hematoxylin and eosin for light microscopy. In paraffin sections, as well as in frozen sections, periodic acid-Schiff (PAS) method for detection of glucan particles was used.

Antibodies

Monoclonal antibodies used for immunohistochemistry were as follows: anti-mouse monoclonal antibodies

Table 1. Antigenic Specificities and Immunoreactive Cells of Monoclonal Antibodies

Monoclonal antibody	Isotype	Immunoreactive cells	References
F4/80	IgG2b	Promonocytes, monocytes, free or tissue-fixed macrophages, Kupffer cells, histiocytes, synovial A cells, microglial cells, phagocytes on periosteal and endosteal bone surfaces, epidermal Langerhans cells	54, 55
ER-MP20	IgG2a	Macrophage colony-forming cells, monoblasts, promonocytes, monocytes, immature macrophages	56, 57
ER-MP58	IgM	Myeloid precursors (granulocyte-macrophage and macrophage colony forming cells), monoblasts, monocytes	56, 57
ER-MP12	IgM	Myeloid precursors (granulocyte-macrophage and macrophage colony-forming cells), monoblasts	56, 57
Thy-1.2	IgG2b	T lymphocytes	58
NLDC-145	IgG2a	Dendritic cells, interdigitating cells, veiled cells, Langerhans cells of the skin	59-61
ERTR-3	IgG2b	Dendritic cells, B cells, macrophages	61
B7-2	IgG2a	B cells, T cells, macrophages, dendritic cells	62
B7-1	IgG2a	Dendritic cells, monocytes, peritoneal macrophages	62
B220	IgG2a	B lymphocytes	63

against mouse macrophages F4/80,^{54,55} against monocytic cells ER-MP20^{56,57} or myeloid macrophage precursor ER-MP58 and ER-MP12,^{56,57} against T lymphocytes Thy-1.2,⁵⁸ against dendritic cells NLDC-145,⁵⁹⁻⁶¹ and against murine major histocompatibility complex II antigen ERTR-3,⁶¹ (purchased from BMA Biomedicals, August, Switzerland); as well as those against CD86 co-stimulatory molecule B7-2, against CD80 co-stimulatory molecule B7-1,⁶² and against murine B lymphocytes B220 (from Pharmingen, San Diego, CA).⁶³ Table 1 shows the antigenic specificities and immunoreactive cells of the monoclonal antibodies used in the present study. To produce a polyclonal antibody against β -glucan, adult Wistar rats received two intravenous injections, 1 week apart of 2 mg of β -glucan. Animals were killed at 1 week after the last injection, and serum was obtained and used as the polyclonal antibody against β -glucan.

Immunohistochemistry

Frozen tissues were cut by a cryostat into 5- μ m-thick sections. After inhibition of endogenous peroxidase activity by the method of Isobe et al,⁶⁴ the indirect immunoperoxidase method using the above mentioned monoclonal antibodies and polyclonal antibody was performed as described previously.⁴⁻⁶ As a secondary antibody, we used anti-rat immunoglobulin-horseradish peroxidase-linked F(ab')₂ fragment (Amersham, Poole, UK). After application of 3,3'-diaminobenzidine, hematoxylin was used for nuclear staining and sections were mounted with malinol. Negative controls underwent the same procedures performed, but the primary antibodies were omitted.

Evaluation of Hepatic Granulomas

On the basis of previous studies,⁴⁻⁶ hepatic granulomas were defined as being composed of more than 10 cells. The number of granulomas per 1 mm² was counted. In each section, 100 granulomas were randomly selected and evaluated.

Autoradiography with [³H]Thymidine

[³H]Thymidine (specific activity, 0.3 to 0.5 MBq/mmol) was purchased from Amersham and stored at 4°C. At 2, 3, 5, 8, 10, 14, and 21 days after zymocel injection, mice were injected intraperitoneally with [³H]thymidine and were killed at 60 minutes after pulse labeling. After immunohistochemical staining with F4/80 and color development by 3,3'-diaminobenzidine, slides were dipped in a Sakura NR-M2 liquid emulsion (Konica, Tokyo, Japan) diluted 1:1 with distilled water, were kept for 7 days in a dark place, and were developed. Cell nuclei with 10 or more grains above the background level were defined to be labeled.²⁻⁶

RT Reaction with Nested PCR

To detect the GM-CSF mRNA, we used the RT reaction, performed as reported previously,^{65,66} with a random primer, nested PCR was done using the following primers. The sequences of outer and inner primers of GM-CSF were as follows: outer primer: sense: 5'-TGAGGAGGATGTGGC-TGCA-3', antisense: 5'-CAGGCACAAAAGCAGCAGTC-3', size of the amplified product, 488 bp; inner primer: sense: 5'-TGTGGTCTACAGCCTCTCAGCAC-3', antisense: 5'-CAAGGGGATATCAGTCAGAAAGGT-3', size of the amplified product, 368 bp (Table 2).

DNA Nick-End Labeling

DNA nick-end labeling was used to detect apoptotic cells within or outside of hepatic granulomas; labeling was performed with the ApopTag detection kit (Intergen, Purchase, NY).⁶⁷ Formalin-fixed paraffin sections were deparaffinized with xylene, and proteins were stripped off by incubation with 20 mg/ml proteinase K (Sigma Chemical, St. Louis, MO) for 15 minutes at room temperature. The tissues were washed in distilled water, and sections were immersed in 3% H₂O₂ solution for 5 minutes to inhibit endogenous peroxidase activity. The sections were exposed to an equilibration buffer and reacted with

Table 2. Sequences of Oligonucleotide Primers Used for RT-PCR

mRNA	Primers	Sequences (5' to 3')	Product (bp)
GM-CSF Outer	Sense	TGAGGAGGATGTGGCTGCA	488
	Antisense	CAGGCACAAAAGCAGCAGTC	
Inner	Sense	TGTGGTCTACAGCCTCTCAGCAC	368
	Antisense	CAAAGGGGATATCAGTCAGAAAGGT	
G3PDH	Sense	GGAAAGCTGTGGCGTGATG	392
	Antisense	CTGTTGCTGTAGCCGTATTC	

G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

terminal deoxynucleotidyl transferase at 37°C for 1 hour, after which the reaction was stopped with stop/wash buffer. Samples were treated with anti-digoxigenin peroxidase conjugate and were incubated for 30 minutes at room temperature. After the sections were washed in phosphate-buffered saline, they were exposed to a peroxidase substrate containing 3,3'-diaminobenzidine. Hematoxylin was used as a counter stain for light microscopy. Positive control samples were prepared from the thymus of BALB/c mice given intraperitoneal injections of dexamethasone.

In Situ Hybridization

The *in situ* hybridization procedure was a modification of the procedure described elsewhere.⁶⁸ Fresh frozen sections, 8- μ m thick, were prepared from the livers of both types of mice at 5 days after zymocel injection and were mounted on silane-coated glass slides. *In situ* hybridization was performed using a mouse GM-CSF oligonucleotide probe cocktail (Funakoshi, Tokyo, Japan). The sections were fixed in 4% paraformaldehyde for 20 minutes, treated with proteinase K, and acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine-HCl buffer (pH 8.0) at room temperature for 10 minutes. After the sections were dehydrated and dried in air, they were treated with hybridization solution containing the probe cocktail and were incubated at 37°C for 24 hours in a humidified chamber. The sections were then washed briefly in 2 $\times$ concentrated standard saline citrate (SSC) (1 $\times$ SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate) and in 50% formamide with 2 $\times$ SSC at 40°C for 30 minutes. After reaction with a digoxigenin-labeled blocking reagent for 1 hour, the sections were exposed to anti-digoxigenin antibody overnight in 4°C. Hybridized digoxigenin-labeled probes were detected by the use of a nucleic acid detection kit (Boehringer-Mannheim, Mannheim, Germany) containing nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate, and toluidine salt, and the sections were kept in a dark place for 24 hours. After the development of color, the sections were rinsed in 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L ethylenediaminetetraacetic acid and were stained with methyl green for nuclear visualization. The specificity of the oligonucleotide probe used for this procedure was verified by comparison with slides treated with hybridization solution without the addition of the probe cocktail and with the slides of GM-CSF-deficient mice.

Electron Microscopy

Livers were taken from GM-CSF-deficient mice and wild-type mice after zymocel injection and were fixed in 2.5% glutaraldehyde and 1% osmium oxide. After fixation, tissues were dehydrated in a graded series of ethanol concentration, were processed in propylene oxide, and were embedded in Epok 812 (Okenshoji, Tokyo, Japan). Sections were cut 1- μ m thick by use of an ultratome (MT-7000 Ultramicrotome, Research and Manufacturing Co. Inc., Tucson, AZ), were stained with toluidine blue, and were observed by a light microscope to detect the presence of apoptotic cells in the granulomas. Ultrathin sections were then cut, stained by lead acetate and uranyl acetate, and studied by use of an H-300 electron microscope (Hitachi, Tokyo, Japan).

Cell Enumeration in Tissues

In immunostained sections, numbers of cells positive for F4/80, ER-MP20, ER-MP58, Thy-1.2, ERTR-3, and B7-2 within and outside of the granulomas were counted per 1-mm² section. In the paraffin sections, numbers of [³H]thymidine-labeled F4/80⁺ cells were counted after autoradiography was performed. In DNA nick-end-labeled paraffin sections, numbers of apoptotic cells were counted within and outside of the granulomas, and their numbers per 1-mm² sections were calculated.

Statistics

Statistical significance of the data was evaluated by analysis of variance with post hoc testing. *P* values < 0.05 were considered significant.

Results

Changes in Numbers of White Blood Cells and Monocytes in Peripheral Blood after Intravenous Injection of Zymocel

After intravenous injection of zymocel, numbers of white blood cells and monocytes increased in peripheral blood of GM-CSF^{-/-} and GM-CSF^{+/+} mice, peaked at 5 days, and then declined (Figure 1, A and B). The numbers of monocytes were slightly lower in GM-CSF^{-/-} mice than in

Figure 1. Changes in numbers of white blood cells (**A**) and monocytes (**B**) in the peripheral blood of GM-CSF^{+/+} and GM-CSF^{-/-} mice after zymocel injection. **A:** Numbers of leukocytes increased in the peripheral blood at 2 days after injection, reached a maximum at 5 days, and then declined in both types of mice. **B:** Numbers of monocytes increased after injection, reached a maximum at 5 days, and then declined in both types of mice.

GM-CSF^{+/+} mice (Figure 1B). However, no significant differences in blood monocyte counts were found between both types of mice. These data indicate that the production of white blood cells including monocytes in bone marrow and their mobilization into peripheral circulation in response to zymocel stimulation are not impaired in GM-CSF^{-/-} mice.

Hepatic Granuloma Formation of GM-CSF^{-/-} Mice after Intravenous Injection of Zymocel

Before zymocel injection, the distribution and localization of F4/80⁺ Kupffer cells in the livers of GM-CSF^{-/-} mice were similar to those of GM-CSF^{+/+} mice. At 1 day after injection, ER-MP20⁺ monocytes infiltrated the hepatic sinusoids of GM-CSF^{+/+} and GM-CSF^{-/-} mice after polymorphonuclear leukocyte infiltration. In GM-CSF^{+/+} mice, F4/80⁺ cell aggregates collected in the hepatic sinusoids at 2 days after injection, and granulomas started to form (Figure 2). The numbers and mean diameters of hepatic granulomas increased, peaked at 10 days, and then declined (Figure 3, A and B). At 2 days after zymocel injection, the development of hepatic granulomas in GM-CSF^{-/-} mice was impaired significantly compared with that in GM-CSF^{+/+} mice (Figure 2A) and granuloma numbers and mean diameters were smaller (Figure 3, A and B). At this stage, the percentages of F4/80⁺ cells and ER-MP20⁺ monocytes per granuloma were significantly reduced in GM-CSF^{-/-} mice (Figure 4). The numbers of ER-MP58⁺ (Figure 5B) or ER-MP12⁺ (data not shown) myeloid precursors in hepatic granulomas were small, and no significant differences in the numbers were found in both types of mice. After daily subcutaneous injection of GM-CSF into GM-CSF^{-/-}

mice, the granuloma numbers and mean diameters recovered to a certain extent, but not completely, to approach those of GM-CSF^{+/+} mice (Figure 3, A and B). Also, the numbers of ER-MP20⁺ monocytes and F4/80⁺ macrophages within the granulomas increased in GM-CSF^{-/-} mice after GM-CSF injection. These data suggest that the development of hepatic granulomas in GM-CSF^{-/-} mice is reduced as a result of the impaired influx of monocytes into the granulomas during the early stage of hepatic granuloma formation.

From 3 to 8 days, the numbers and mean diameters of granulomas were smaller in GM-CSF^{-/-} mice compared with those in the GM-CSF^{+/+} mice, however, differences were not statistically significant. After 10 days, the numbers of granulomas decreased rapidly in GM-CSF^{-/-} mice, accompanied by a significant reduction in mean granuloma diameter at 10 days and in the percentages of F4/80⁺ macrophages from 8 days and thereafter. During this period, the numbers and mean diameters of granulomas, as well as the percentages of F4/80⁺ macrophages, were not significantly reduced in GM-CSF-treated GM-CSF^{-/-} mice compared with the GM-CSF^{+/+} mice. These results suggest that GM-CSF deficiency causes a significant reduction of hepatic granuloma formation at or after 10 days after zymocel injection.

Local Proliferation and Death of Macrophages during Hepatic Granuloma Formation

Figure 6 shows infiltration of [³H]thymidine-labeled F4/80⁺ macrophages within hepatic granulomas of GM-CSF^{+/+} mice and GM-CSF^{-/-} mice with or without daily GM-CSF administration. The proliferative potential of macrophages within granulomas of GM-CSF^{-/-} mice

Figure 2. Granuloma formation and infiltration of F4/80⁺ and ER-MP20⁺ cells in the livers of homozygous mutant (GM-CSF^{-/-}) and wild-type (GM-CSF^{+/+}) mice after zymocel injection. At 2 days after injection, granuloma formation and infiltration of F4/80⁺ cells in the liver is less marked in the GM-CSF^{-/-} mouse (A) than in the GM-CSF^{+/+} mouse (B). At 10 days, hepatic granulomas were fewer in the GM-CSF^{-/-} mouse (C) than in the GM-CSF^{+/+} mouse (D). At 2 days after injection ER-MP20⁺ cells in the granulomas are fewer in the GM-CSF^{-/-} mouse (E) than in the GM-CSF^{+/+} mouse (F). At 10 days after injection, ER-MP20⁺ cells in the granulomas were less marked in the GM-CSF^{-/-} mouse (G) than in the GM-CSF^{+/+} mouse (H). Indirect immunoperoxidase method with F4/80 (A–D) or for ER-MP20 (E–H). Original magnification, $\times 240$.

was significantly lower at 2 days after zymocel injection (Figure 7A) compared with that of GM-CSF^{+/+} mice (Figure 7B). GM-CSF-treatment of GM-CSF^{-/-} mice increased the proliferative potential of granuloma macrophages to the level of GM-CSF^{+/+} mice from 5 days after zymocel injection. These data indicate that GM-CSF deficiency causes a significant reduction in proliferative capacity of macrophages in the early stage of zymocel-induced hepatic granuloma formation, suggesting that

impaired development of hepatic granulomas in the early stage is partly because of a reduced proliferative potential of granuloma macrophages in GM-CSF^{-/-} mice.

Figure 8 shows the numbers of apoptotic cells within (Figure 8A) and outside of (Figure 8B) hepatic granulomas in GM-CSF^{-/-} and GM-CSF^{+/+} mice. At the same time that granuloma number and size began to be reduced more rapidly in GM-CSF^{-/-} mice than in GM-CSF^{+/+} mice, the numbers of apoptotic cells per 1 mm²

Figure 3. Changes in number (A) and mean diameter (B) of granulomas in livers of homozygous mutant (GM-CSF^{-/-}) mice, wild-type (GM-CSF^{+/+}) mice, and mutant GM-CSF^{-/-} mice given GM-CSF (GM-CSF^{-/-} + GM-CSF) after zymocel injection. Data points represent three mice.

section were significantly increased in GM-CSF^{-/-} mice compared with GM-CSF^{+/+} mice ($P < 0.05$). Electron microscopy demonstrated condensed nuclear chromatin, fragmented nuclei, and formation of apoptotic bodies in hepatic granulomas, particularly in GM-CSF^{-/-} mice at 8 days after zymocel injection (Figure 7G). Daily administration of GM-CSF to GM-CSF^{-/-} mice reduced the numbers of apoptotic cells within and outside of the granulomas at 10 days after zymocel injection, nearly to the level of the wild-type mice (Figure 8, A and B).

These results show that GM-CSF deficiency increases apoptosis in hepatic granulomas, particularly during the late stage of granuloma formation, and induces a rapid disappearance of the granulomas in the homozygous mutant GM-CSF^{-/-} mice.

Digestive Capacity of Granuloma Macrophages for Zymocel

To examine the digestive capacity of macrophages for zymocel during hepatic granuloma formation in GM-CSF^{-/-} and GM-CSF^{+/+} mice, the numbers of glucan particles within granuloma macrophages were counted by a double-staining method (PAS reaction and immunostaining with a polyclonal antibody against β -glucan): zymocel (β -glucan) particles were clearly stained reddish purple by the PAS reaction and brown by immunostaining with the anti- β -glucan polyclonal antibody. The numbers of glucan particles were counted, although because glucan particles were digested and degraded within the macrophages with subsequent loss of particular shape, they cannot be counted within macrophages, particularly in the late stage of hepatic granuloma formation. Figure 9 shows changes in the numbers of granuloma macrophages ingesting glucan during hepatic granuloma formation in GM-CSF^{-/-} and GM-CSF^{+/+} mice. The num-

bers of cells increased to a peak at 8 days (Figure 7, C and D) and declined thereafter in both types of mice. These data suggest that the uptake and degradation of zymocel particles by granuloma macrophages were not different in GM-CSF^{-/-} and GM-CSF^{+/+} mice.

Changes in Numbers of Lymphocytes during Hepatic Granuloma Formation

Small numbers of lymphocytes were usually found in the livers of GM-CSF^{+/+} and GM-CSF^{-/-} mice. After zymocel injection, lymphocytes infiltrated the livers of both types of mice. Most lymphocytes were Thy-1.2⁺ T cells, only a few B220⁺ B cells were present in and around the granulomas. The numbers of T cells per 1 mm² increased within hepatic granulomas in parallel with increases in the numbers and diameters of hepatic granulomas (Figure 5A). In addition, natural killer cells were observed in the hepatic granulomas (data not shown). However, the numbers of T cells per 1 mm² within and outside of the granulomas were lower in GM-CSF^{-/-} mice than in GM-CSF^{+/+} mice at 8 days after zymocel injection; this reduction in the GM-CSF^{-/-} mice was not significant statistically.

Activation of Macrophages during Hepatic Granuloma Formation

Figure 10A shows changes in the number of ERTR-3⁺ cells in hepatic granulomas of GM-CSF^{-/-} and GM-CSF^{+/+} mice after zymocel injection. Numbers of these cells increased in parallel with increases in the number and size of granulomas, peaked at 8 days in both types of mice, and declined thereafter. GM-CSF^{-/-} mice had a

Figure 4. Changes in numbers and percentages of F4/80⁺ cells and ER-MP20⁺ monocytes within (A and C) and outside of (B and D) granulomas in livers of homozygous mutant (GM-CSF^{-/-}) mice, wild-type (GM-CSF^{+/+}) mice, and GM-CSF^{-/-} mice given GM-CSF (GM-CSF^{-/-} + GM-CSF) after zymocel injection. Data points represent three mice.

smaller number of ERTR-3⁺ cells at 2 days after injection, but this difference was not statistically significant. Figure 10B shows the numbers of co-stimulatory molecule B7-2⁺ cells in hepatic granulomas of both types of mice after zymocel injection. B7-2⁺ cells were present throughout granuloma formation and their numbers peaked at 8 days after injection in both types of mice, but with no statistical difference in the two types of mice. A few B7-1⁺ cells were present in hepatic granulomas of both types of mice from 2 to 10 days after injection (data not shown). NLDC-145⁺ dendritic cells were present in granulomas of both types of mice from 2 days to 10 days after injection (data not shown). These data suggest that activation of macrophages during zymocel-induced hepatic granuloma formation is not impaired in GM-CSF^{-/-} mice.

Distribution of GM-CSF mRNA-Expressing Cells in Livers of GM-CSF^{-/-} and GM-CSF^{+/+} Mice after Zymocel Injection

To detect GM-CSF mRNA expression, the distribution of GM-CSF mRNA-expressing cells in liver tissue sections was examined by *in situ* hybridization using a GM-CSF oligonucleotide probe cocktail. Purplish blue-stained positive signals were detected in livers of GM-CSF^{+/+} mice, in the endothelial cells of lymph vessels (Figure 11A), some granuloma cells and hepatocytes (Figure 11B), and endothelium of blood vessels (Figure 11C). However, GM-CSF^{-/-} mice showed no expression of this cytokine in any cells in livers throughout the experimental

Figure 5. Infiltration of Thy1.2⁺ cells (A) and ER-MP 58⁺ cells (B) in hepatic granulomas of homozygous mutant (GM-CSF^{-/-}) mice, wild-type (GM-CSF^{+/+}) mice, and GM-CSF^{-/-} mice given GM-CSF (GM-CSF^{-/-} + GM-CSF). Data points represent three mice.

period (Figure 11D). By RT-nested PCR, the expression of GM-CSF mRNA was proved in the livers of GM-CSF^{+/+} mice before and after zymocel injection and not in those of GM-CSF^{-/-} mice throughout the experimental period (Figure 11E).

Discussion

In the present investigation, hepatic granuloma formation in GM-CSF-deficient mice, but not wild-type mice, was

significantly delayed until 5 days after zymocel injection, and the numbers and diameters of hepatic granulomas decreased more rapidly after 10 days in the homozygous mutant (GM-CSF^{-/-}) mice than in the wild-type (GM-CSF^{+/+}) mice. In addition, the percentages of ER-MP20⁺ monocytes and F4/80⁺ macrophages within hepatic granulomas were significantly reduced in the GM-CSF^{-/-} mice compared with the GM-CSF^{+/+} mice, indicating that the influx of monocytes into the granulomas and their differentiation into macrophages are impaired in GM-CSF^{-/-} mice. By RT-nested PCR, the expression of GM-CSF mRNA was proved in the GM-CSF^{+/+} mice and not in the GM-CSF^{-/-} mice.

In GM-CSF^{-/-} mice, the early stage of hepatic granuloma formation was impaired, in agreement with results from previous studies of mice depleted of blood monocytes by ⁸⁹Sr,^{4,5} osteopetrosis (op/op) mice,³ and mice depleted of Kupffer cells by administration of liposome-entrapped dichloromethylene diphosphonate.² In ⁸⁹Sr-induced monocytopenic mice and M-CSF-deficient op/op mice, the deficiency of blood monocytes is the major cause of markedly impaired hepatic granuloma formation. In op/op mice, the differentiation of monocytes into macrophages is severely impaired because of the M-CSF deficiency. The depletion of Kupffer cells in mice treated with liposome-entrapped dichloromethylene diphosphonate results in impaired production of monocyte chemoattractant protein (MCP-1), tumor necrosis factor (TNF)- α , and interferon (IFN)- γ , which induce monocyte migration into hepatic granulomas and differentiation of monocytes into macrophages, as well as macrophage activation within the granulomas.² However, data presented in previous studies,^{47,48} have indicated that the development of hematopoietic cells in the bone marrow and peripheral blood of GM-CSF^{-/-} mice was

Figure 6. Changes in infiltration of [³H]thymidine-labeled F4/80⁺ cells in granulomas in livers of homozygous mutant (GM-CSF^{-/-}) mice, wild-type (GM-CSF^{+/+}) mice, and GM-CSF^{-/-} mice given GM-CSF administration (GM-CSF^{-/-} + GM-CSF). Data points represent three mice.

Figure 7. [³H]Thymidine-labeled F4/80⁺ macrophages in hepatic granulomas of GM-CSF^{-/-} mice (A) and wild-type mice (B) at 2 days after zymocel injection; PAS and anti-glucan double staining of hepatic granulomas of both types of mice at 8 days after zymocel injection (C and D); DNA nick-end labeling of apoptosis in GM-CSF^{-/-} mice (E) and wild-type mice (F) at 8 days after zymocel injection; and electron microscopy of apoptotic bodies in the granulomas of GM-CSF^{-/-} mice (G). Staining of [³H]thymidine-labeled F4/80⁺ cells were fewer in GM-CSF^{-/-} mice (A) than GM-CSF^{+/+} mice (B) at 2 days after injection. [³H]Thymidine autoradiography and immunostaining with F4/80. Double staining indicated no difference in both types of mice (C and D). Double staining with PAS reaction and indirect immunoperoxidase method using anti-glucan polyclonal antibody. Apoptotic cells (stained brown) were more numerous in hepatic granulomas at 8 days in GM-CSF^{-/-} mice (E) compared with the wild-type mice (F). DNA nick-end labeling with *in situ* apoptosis detection kit. Apoptotic bodies were seen in the phagocytic vacuole of a macrophage at 8 days after zymocel injection in GM-CSF^{-/-} mice (G). Transmission electron microscopy. Original magnification, ×720 (A and B), ×360 (C–F), ×12,000 (G).

not impaired and that the development and distribution of Kupffer cells were normal, as shown in the current study. In the GM-CSF^{-/-} mice studied here, the production of

white blood cells and monocytes in bone marrow and their mobilization into peripheral blood in response to zymocel injection were not impaired. In the early stage of

Figure 8. Numbers of apoptotic cells within (**A**) and outside of (**B**) granulomas in livers of homozygous mutant (GM-CSF^{-/-}) mice, wild-type (GM-CSF^{+/+}) mice, GM-CSF^{-/-} mice given GM-CSF (GM-CSF^{-/-} + GM-CSF) after zymocel injection. Data points represent three mice.

granuloma development in the liver of GM-CSF^{-/-} mice, the influx of ER-MP20⁺ monocytes into the liver was not impaired. However, monocyte migration into granulomas and monocyte aggregation were markedly impaired in GM-CSF^{-/-} mice, accompanied by a significant reduction in the proliferative capacity of granuloma macrophages, as shown by their [³H]thymidine-labeling rates. Because GM-CSF is known to induce *in vitro* proliferation of macrophages^{23,24} and chemotaxis of monocytes,^{38,40} the reduced proliferative potential of macrophages and the impaired influx of monocytes into hepatic granulomas of GM-CSF^{-/-} mice may be ascribed to total deficiency of GM-CSF *in loco*.

GM-CSF is essential for the differentiation and survival, as well as the proliferation, of macrophages.^{23,24,41} In GM-CSF^{-/-} mice, the influx of monocytes into hepatic

granulomas and monocyte differentiation into macrophages in response to zymocel stimulation are impaired and these impairments are thought to be caused by GM-CSF deficiency. The rapid reduction in numbers of hepatic granulomas with their eventual disappearance in GM-CSF^{-/-} mice from 10 days after zymocel injection is also essentially because of the lack of GM-CSF production in the liver: granuloma macrophages could not survive in GM-CSF^{-/-} mice, and apoptotic cells were more numerous in the granulomas of GM-CSF^{-/-} mice at 10 days after zymocel injection than in GM-CSF^{+/+} mice, as demonstrated by electron microscopy. These findings suggest that GM-CSF is important for the survival of macrophages and for the maintenance of the late stage of hepatic granulomas.

Zymocel is composed of β -glucan, an intense stimulant of macrophages,¹⁶ neutrophils,¹⁷ and natural killer cells,¹⁸ and is taken up by macrophages via the receptor for β -glucan,¹⁹ which was recently demonstrated to be a lectin domain of complement receptor type 3 (CR3 or CD11b/CD18),²⁰ exposing an I domain neopeptide for binding to β -glucan.⁶⁹ In contrast, GM-CSF stimulates macrophages to induce increases in protein tyrosine phosphorylation through activation of multiple tyrosine kinases such as Src-like tyrosine kinases, Fes, and p^{97, 42-44}. In this way, signal transduction and production of GM-CSF by macrophages and lectin-dependent CR3-mediated signal transduction of β -glucan through CD11b/CD18 are considered to be unrelated. Our preliminary study has demonstrated expressions of proinflammatory cytokines such as MCP-1, TNF- α , and IFN- γ mRNAs in the livers of GM-CSF^{-/-} mice after zymocel injection (data not shown), a finding which seems to be induced by macrophages in response to glucan stimulation. In contrast, our preliminary study has also demonstrated expression of M-CSF, IL-1, IL-3, or IL-5 mRNAs in the liver of GM-CSF^{+/+} mice before and after zymocel

Figure 9. Numbers of glucan-ingesting cells in hepatic granulomas of homozygous mutant (GM-CSF^{-/-}) mice and wild-type (GM-CSF^{+/+}) mice.

Figure 10. Changes in percentages of ERTR-3⁺ cells (**A**) and B7-2⁺ cells (**B**) in hepatic granulomas of homozygous mutant (GM-CSF^{-/-}) mice and wild-type (GM-CSF^{+/+}) mice after zymocel injection. Numbers of these cells increased with increases in numbers and diameters of hepatic granulomas, peaked at 8 days in both types of mice, and then declined.

Figure 11. Distribution of cells expressing GM-CSF mRNA in livers of GM-CSF^{+/+} (**A–C**) and GM-CSF^{-/-} (**D**) mice and the expression of GM-CSF mRNA in both types of mice (**E**) after zymocel injection as detected by *in situ* hybridization (**A–D**) and RT-nested PCR (**E**). Endothelial cells of lymph vessel (**A**), some granuloma cells (**arrow**) and hepatocytes (**arrowhead**) (**B**), and endothelium of blood vessel (**C**) stained purplish blue, a positive signal, in GM-CSF^{+/+} mice. There is no hybridization signal for GM-CSF mRNA in the liver of GM-CSF^{-/-} mice (**D**). *In situ* hybridization with methyl green counterstain. GM-CSF is expressed in the livers of GM-CSF^{+/+} mice and not in those of GM-CSF^{-/-} mice throughout the experimental period (**E**). Each line shows 1-kb marker (m), 0 day, 2 days, 3 days, 5 days, 8 days, 10 days, 14 days, and 21 days after zymocel injection. Original magnification, $\times 200$ (**A–D**).

injection (data not shown), a finding that reflects constitutive production of these cytokines, although GM-CSF^{-/-} mice fail to produce GM-CSF in tissues including the liver. In mice infected with *L. monocytogenes*, expression of IFN- γ , TNF- α , IL-10, and GM-CSF was detected in the early stage of hepatic granuloma formation.¹⁵ On the basis of this information, it is thought that the proinflammatory cytokines such as M-CSF, IL-1, IL-3, IL-5, MCP-1, TNF- α , and IFN- γ may induce differentiation, maturation, activation, and proliferation of macrophages within hepatic granulomas of GM-CSF^{-/-} and GM-CSF^{+/+} mice.

T cells also play important roles in macrophage activation during the hepatic granuloma formation. Previous studies revealed marked delay in hepatic granuloma formation in response to glucan stimulation, in immunodeficient mice such as nude mice or scid mice, and T cells are severely deficient in both types of mice.⁷ In contrast, previous studies in glucan-induced hepatic granuloma formation in xid mice, a model of impaired B cell proliferation and differentiation, showed that T cell numbers in liver granulomas increased throughout the experiment, granuloma formation peaked at 5 days and rapidly declined thereafter and that uptake and degradation of glucan by granuloma macrophages were accelerated, suggesting that increases in the number of T cells within the granulomas induced a more marked activation of macrophages.⁷ However, in GM-CSF^{-/-} mice studied here, the numbers of T cells in hepatic granulomas were lower than those in GM-CSF^{+/+} mice, although this reduction was not significant statistically. Expression of IFN- γ and TNF- α mRNAs in the GM-CSF^{-/-} mice was not impaired, and expression of the CD80 and CD86 co-stimulatory molecules B7-1 and B7-2 in granuloma cells was not reduced in the GM-CSF^{-/-} mice. In the processes of glucan uptake by macrophages via CR3, cyclooxygenase (prostaglandin E₂) and 5'-lipoxygenase (leukotriene C4) pathways are used for the production of eicosanoids⁷⁰ and are independent of macrophage activation through GM-CSF signal transduction pathways. These data suggest that activation of granuloma macrophages is not impaired in the GM-CSF^{-/-} mice and that β -glucan can stimulate and activate macrophages through pathways different from those of GM-CSF signal transduction in mutant mice. The present study also revealed that the uptake and degradation of glucan particles by granuloma macrophages were not impaired in the mutant mice compared with the GM-CSF^{+/+} mice.

In the present study, daily administration of GM-CSF into GM-CSF^{-/-} mice induced a recovery of impaired hepatic granuloma formation nearly to the level of GM-CSF^{+/+} mice, accompanied by increased numbers of F4/80⁺ macrophages and ER-MP20⁺ monocytes within hepatic granulomas in the early stage and by decreased numbers of apoptotic cells at 10 days after injection. These data provide evidence to the facts that GM-CSF is essential for inducing chemotaxis of monocytes into granulomas, their differentiation into macrophages, and survival of macrophages within the granulomas. Previous studies showed that GM-CSF is constitutively produced by hepatocytes, Kupffer cells, fibroblasts, and sinusoidal endothelial cells,⁷¹⁻⁷⁷ in agreement with the results of the

present study that the *in situ* hybridization method showed positive signals in vascular endothelial cells, hepatocytes, and granuloma cells in the liver of GM-CSF^{+/+} mice. However, GM-CSF is not demonstrated at message level in the liver tissues of GM-CSF-deficient mice.

In conclusion, the data presented in this study provide evidence that GM-CSF participates in the proliferation, differentiation, and survival of macrophages within the hepatic granulomas, and induces monocyte chemotaxis and migration into the granulomas.

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