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Colony-Stimulating Factor 1-Dependent Resident Macrophages Play a Regulatory Role in Fighting *Escherichia coli* Fecal Peritonitis

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Osteopetrotic *op*/*op* mice have less than 5% of the normal number of macrophages in the peritoneal cavity (W. Wiktor-Jedrzejczak, A. Ahmed, C. Szczylik, and R. R. Skelly, J. Exp. Med. 156:1516–1527, 1982). Fecal peritonitis was induced by intraperitoneal injection of 0.5 ml of 5% autoclaved feces in saline along with *Escherichia coli* grown from feces of mice of the same colony and added in doses ranging between 10 and 10^6 CFU. Such infection led to a septic shock and either was lethal within 24 h or became cured without additional treatment of the mice. The *op*/*op* mice survived administration of 30-times-smaller doses of bacteria compared with their normal littermates. Analysis of the kinetics of cellular changes in the peritoneal cavity associated with such infection revealed that this increased susceptibility of macrophage-deficient mice cannot be explained by a direct role of macrophages in combating the infection. Instead, it appeared that the increased susceptibility to fatal fecal peritonitis was most likely due to delayed and impaired recruitment of neutrophils to the site of infection in mutant mice. The increased susceptibility of the *op*/*op* mice to *E. coli* fecal peritonitis was not due to their possible increased sensitivity to endotoxin, since the mutant mice tolerated lipopolysaccharide doses more than twice those tolerated by control littermates. On the other hand, their susceptibility to exogenous tumor necrosis factor alpha and interleukin-1α was increased. Both mutant *op*/*op* and control mice were able to survive secondary challenge with 10^6 *E. coli* cells (administered along with feces) lethal for both types of mice on primary challenge. These data suggest that colony-stimulating factor 1-dependent resident peritoneal macrophages play a role in controlling primary infection by recruiting neutrophils and are not required for efficient response to secondary infection.

Macrophages are cells classically held responsible for fighting bacterial infections (17). This notion has never been confirmed or investigated in studies using animals congenitally deprived of those cells. Some time ago, we identified a mouse mutant at the *op* locus as having a profound macrophage deficiency (20). Subsequent studies have shown that this deficiency is generalized but differential, with some organ locations being affected more than others (5, 12, 20, 23). The most severe deficiency is in the peritoneal cavity, where *op*/*op* mice have less than 95% of the normal number of macrophages (20, 23, 24). The macrophage deficiency in *op*/*op* mice is due to the complete absence of colony-stimulating factor 1 (CSF-1; also known as M-CSF) (21), a major growth factor for macrophages, which in turn is due to the inactivating mutation in a gene for this factor (26). Therefore, the *op*/*op* mouse is a unique genetically defined model of macrophage deficiency that can be used to investigate the role of macrophages in defense against bacterial infections.

For the initial study, we selected a model of infection which mimics the classical clinical problem (7) and mirrors the condition that most severely affects mutant mice: fecal peritonitis (9) with wild-type *Escherichia coli*, which leads to a death from septic shock (1). Additionally, this model is convenient because it has known, testable, and easily available mediators such as lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), and interleukin-1α (IL-1α) (6, 11).

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The aim of this study was to determine whether *op*/*op* mice are more susceptible than control mice to this infection and whether the time course of cellular changes induced by such infection differs between *op*/*op* and control mice. Additionally, we tested the *op*/*op* mice for their responses to LPS and to recombinant human TNF-α and IL-1α.

MATERIALS AND METHODS

Mice. (C57BL/6 × C3H/FeB)F_2 *op*/+ breeding pairs were purchased from Jackson Laboratory (Bar Harbor, Maine). A subline of C3H mice which contributes to this background is not resistant to endotoxin (15). Subsequent breeding of *op*/*op* and control weight-matched +/+ mice was carried out by selecting and mating *op*/+ and +/+ animals, respectively. Mutant *op*/*op* mice were identified by the absence of incisors and maintained as described previously (22). Equal numbers of 4- to 6-month-old male mice were used for subsequent testing. Because weight-matched controls were used throughout, +/+ mice were usually 2 months younger than *op*/*op* mice. All animal care and use was in strict accordance with the International Guiding Principles for Biomedical Research Involving Animals.

Preparation of bacterial inoculate. Nonpathogenic *E. coli* was isolated from feces of mice of our *op*/*op* breeding colony and identified by using standard methods at the International Escherichia and Klebsiella Centre (World Health Organization) at Statens Seruminstitut, Copenhagen, Denmark. The serotype determined was O13:K^-:H11; the bacteria were hemolysin negative, fimbria negative, and Vero cytotoxin negative. A large batch of bacteria was grown, aliquoted, stored frozen at −80°C, and used in all subsequent experiments, which were performed within 3 months of isolation of bacteria. Additionally, it was confirmed on two separate occasions that *E. coli* isolated from the same mouse colony in subsequent months belonged to the same nonpathogenic serotype. The final inoculum consisted of 0.5 ml of a 0.5% suspension of autoclaved feces in saline and a defined number of bacteria (10-fold dilutions) and was injected intraperitoneally (i.p.). This model of infection was chosen because it closely resembles fecal peritonitis occurring in clinical setting (7).

Observation of infected mice. In experiments testing survival, injected mice were observed at hourly intervals for the first 48 h and thereafter daily for up to 2 months. The exact time of death was determined. Some animals immediately

TABLE 1. Survival of macrophage-deficient *op*/*op* and control +/+ mice receiving various doses of *E. coli* in addition to sterilized feces in order to induce fecal peritonitis

Dose of *E. coli* injected i.p.	No. of mice surviving[a]/no. injected	
	+/+	*op*/*op*
10^6	0/10	Not tested
10^5	0/17	0/6
10^4	7/14	0/6
10^3	11/12	1/8[b]
10^2	10/10	9/10
10	10/10	5/5

[a] Final survival evaluated 6 days after injection; mice were without signs of disease.

[b] Calculated LD_{50}s of 10^4 for +/+ mice and 3.2×10^2 for *op*/*op* mice (31-fold difference).

after death were subjected to peritoneal lavage, and/or some of their organs were collected for histology. In experiments testing cellular kinetics, groups of injected mice were sacrificed at appropriate times and subjected to peritoneal lavage in order to determine the presence of various cell types in peritoneal cavity and the presence of bacteria in lavage fluid. The presence of macrophages, lymphocytes, and neutrophils was assayed either by direct counting in a hemocytometer or by evaluation of Wright's-stained cytospin preparations.

Endotoxin treatment. For determining the lethal dose of endotoxin for the +/+ and *op*/*op* mice, groups of 5 to 10 mice of each genotype were injected i.p. with 2, 2.5, 3.4, 5, 6.5, 10, 15, and 30 μg of *E. coli* endotoxin (*E. coli* LPS, 0111:B4; Difco, Detroit, Mich.) per g of body weight, and the number of mice surviving >2 weeks was recorded.

Recombinant cytokine administration. Since LPS exerts its effects largely through mediators of the initial step of acute-phase response, such as TNF-α and IL-1α (16), the direct sensitivity of the *op*/*op* mice to these mediators was also evaluated. Human recombinant TNF-α was kindly provided by Marek Kwinkowski, Center for Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, Poland. The sterile powder was reconstituted and diluted in 0.1% albumin in phosphate-buffered saline. Animals were treated i.p. with single doses of between 0.08 and 40 μg/g of body weight. Recombinant human IL-1α, obtained from Peter Lomedico (Hoffmann-La Roche, Nutley, N.J.), was administered i.p. at a dose of 2.5 μg per mouse per day for 3 days, a schedule developed to evaluate the effects of this molecule on bone resorption (4). Furthermore, in some experiments highly purified recombinant CSF-1 (8) was obtained from Chiron Corporation, Emeryville, Calif., and administered by i.p. injection, using a daily dose of 10^5 U per mouse (24) in order to obtain *op*/*op* mice with reconstituted peritoneal macrophage population.

Statistics. The 50% lethal dose (LD_{50}) was calculated as described by Reed and Muench (14). Means, standard deviations, and standard errors were calculated, and the differences between means were evaluated by using the Student *t* test. Differences in the numbers of mice surviving administration of TNF-α and IL-1α as well as of mice surviving secondary challenge with *E. coli* were evaluated by using the Σ^2 test.

RESULTS

Course of *E. coli* peritonitis in *op*/*op* and control mice. Injection of neither saline, feces suspension alone, nor bacterial suspension (up to 10^6 CFU) alone induced any visible change in behavior or lethality in mutant or control mice. Therefore, the data from these mice are not shown. Animals injected with *E. coli* along with feces began to look sick about 3 h after infection and gradually became lethargic and hypothermic, developed diarrhea, and began to die about 12 h postinjection. There was no clear-cut relationship between the dose of bacteria injected and time of death. Mice that survived 24 h postinjection gradually recovered, and there were no late deaths with the exception of two *op*/*op* mice that died 44 and 46 h after injection. Control mice challenged with lethal doses of *E. coli* died significantly earlier (16 ± 2 h) than similarly treated *op*/*op* mice (21 ± 3 h; $P < 0.001$).

As shown in Table 1, there was a marked 31-fold difference in survival (LD_{50}) of mutant *op*/*op* mice compared with control mice. Most of the mutant mice survived infection with ≤100 CFU, while most of the normal littermates survived infection with 1,000 CFU, and 50% of them survived infection with 10,000 CFU, administered together with autoclaved feces.

To determine which cellular changes occurring in the peritoneal cavities of infected mice were responsible for these differences, pairs of mutant and control mice infected with 100 CFU were sacrificed and analyzed every hour for 48 h and then every 4 h for 80 h. After identification of critical time points, groups of five mutant and control mice were sacrificed and analyzed for each of these time points.

As shown in Fig. 1, macrophages were nearly absent in peritoneal lavage of *op*/*op* mice initially and for the first 17 h after infection. Unexpectedly, later these cells began to appear in large numbers, reaching at 52 h postinfection several million per lavage. The kinetics in control mice was similar except that macrophages initially present in lavage decreased to very low numbers within 1 h postinfection. They began to reappear about 20 h postinfection and, as in the mutant mice, reached a peak at 52 h, with the number being approximately three times higher than in the *op*/*op* mice.

These data show that the *op*/*op* mice can generate large numbers of macrophages within 48 h under stress conditions, although the magnitude of this response is smaller than in control mice. However, these differences were observed at a time when the outcome of the infection was already known, that is, when animals were already recovering from the infection. Therefore, it is unlikely that the difference in the macrophage kinetics was directly responsible for the more severe course of infection in mutant mice.

Some differences in the lymphocyte kinetics at certain time points have been observed between the *op*/*op* and control mice, which had a reduced lymphocyte increase at 9 h postinfection. However, no lymphocyte effector mechanisms that can be activated in such a short time are known, and this makes the differences in lymphocyte recruitment very unlikely as an explanation for the differences in lethality.

In contrast to the behavior of macrophages and lymphocytes, there was a difference in the kinetics of appearance of neutrophil granulocytes in peritoneal cavities of *op*/*op* and control mice that was observed at a time relevant to survival. In control mice, a large number of neutrophils began to appear in the peritoneal cavity starting at 3.5 h postinfection, reaching very high levels of 10 million and more per lavage at 4.5 h postinfection (Fig. 1). In contrast, in the *op*/*op* mice, this influx of neutrophils to the peritoneal cavity was delayed for about 1 h, and the final magnitude was three to four times lower than in control mice. Therefore, this difference in the kinetics of neutrophil influx to the peritoneal cavity in infected mice corresponded well with the difference in the course of infection and could likely be responsible for the latter difference.

In an additional experiment, 10 *op*/*op* mice were pretreated i.p. for 10 days with 10^5 U of human recombinant CSF-1 (24) per mouse per day and subsequently challenged with 10^3 *E. coli* along with feces. Five mice were sacrificed 4 h later for evaluation of peritoneal lavage, and five were left for evaluation of survival. A similar number of *op*/*op* mice challenged with *E. coli* without CSF-1 pretreatment was also evaluated. CSF-1-pretreated *op*/*op* mice had $(6.5 \pm 1.4) \times 10^6$ neutrophils in peritoneal lavage, compared with $(1.1 \pm 0.6) \times 10^6$ neutrophils in peritoneal lavage of *op*/*op* mice that were not pretreated with CSF-1. Furthermore, all CSF-1-pretreated *op*/*op* mice survived challenge with *E. coli* (evaluated after 1 week), while all *op*/*op* mice that did not receive CSF-1 died within 24 h after challenge. These data provide an additional argument for the role of neutrophil influx mediated by CSF-1-induced cells in resistance to *E. coli* fecal peritonitis.

Alternatively, increased mortality of *op*/*op* mice could be due to their eventual increased susceptibility to endotoxin released by *E. coli* or mediators induced by endotoxins such as

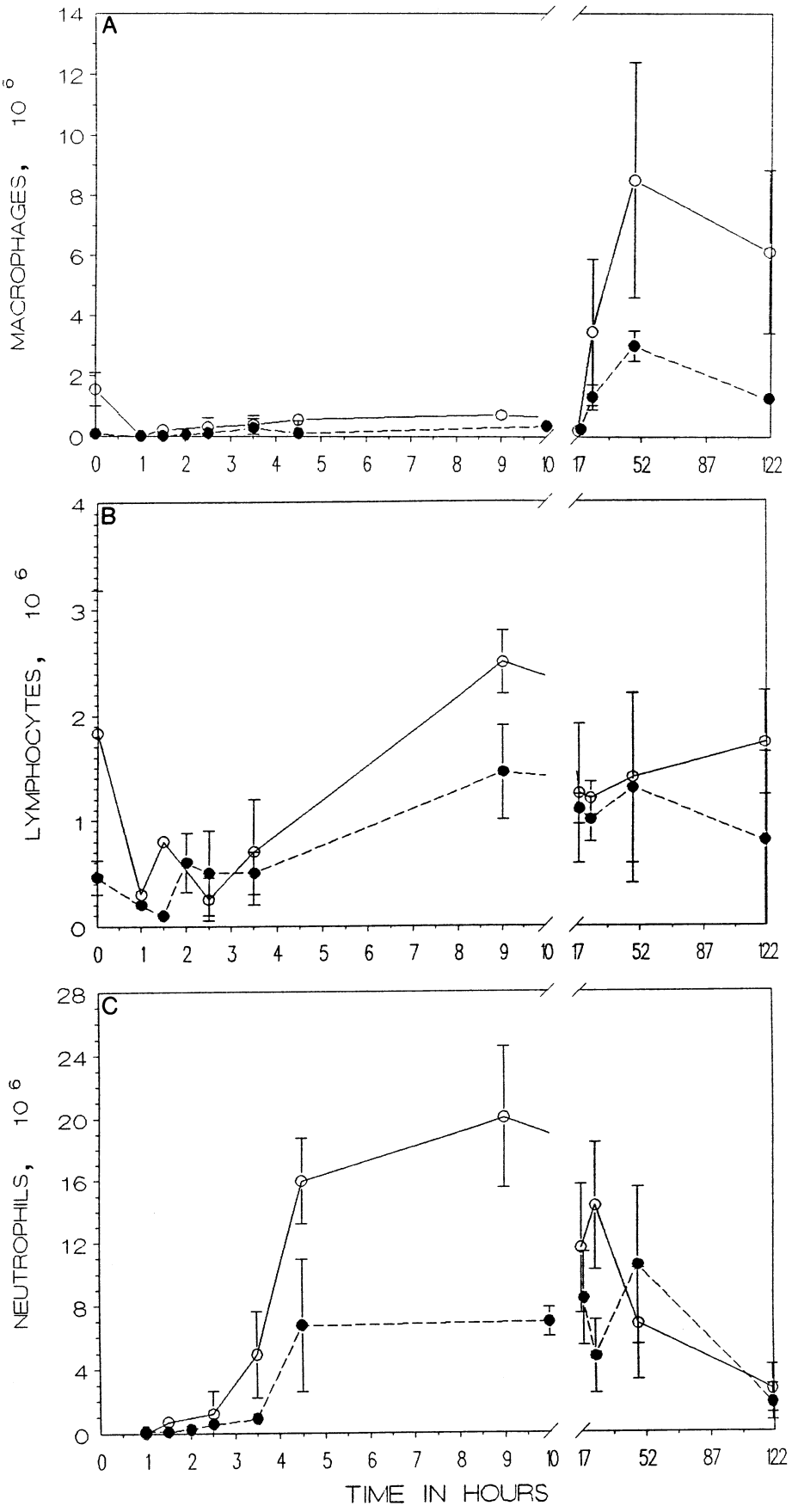

FIG. 1. Kinetics of changes in the number of cells in peritoneal lavage fluid of *op/op* mice (closed circles) and weight-matched +/+ controls (open circles) following i.p. injection of 100 *E. coli* CFU and 0.5 ml of a 5% suspension of autoclaved feces. (A) Kinetics of changes in macrophage number; (B) kinetics of changes in lymphocyte count; (C) kinetics of changes in neutrophil number. Data are means ± standard deviations of data from five mice per point.

TABLE 2. Survival of macrophage-deficient *op*/*op* and control +/+ mice following injection of LPS

LPS (μg/g of body wt)	No. of mice surviving/no. treated[a]	
	+/+	*op*/*op*
30	0/5	0/5
15	0/5	0/5
10	0/5	1/6
6.5	0/6	5/6
5	0/6	5/5
3.4	1/6	5/5
2.5	9/10	5/5
2	10/10	5/5

[a] Male mice with an average weight of 20 g (4 to 6 months old) were used. Calculated LD_{50}s were 3.0 μg/g for +/+ mice and 8.3 μg/g for *op*/*op* mice (2.7-fold difference).

TNF-α and IL-1α. This possibility was examined in subsequent experiments.

Responses of *op*/*op* and control mice to endotoxin. Death resulting from endotoxin injection occurred in mice within 50 h of LPS injection and was preceded by signs of immobility and ruffed fur typical of postendotoxin lethality. As shown in Table 2, doses of >10 μg/g resulted in death of all +/+ and *op*/*op* mice. Of interest was the observation that while almost all *op*/*op* mice survived the challenge at doses of 6.5 μg/g and lower, doses of 6.5 and 5 μg/g were lethal for all +/+ mice, and only one of four +/+ mice receiving 3.4 μg of LPS per g survived. Calculated LD_{50}s of LPS were 3.0 μg/g for +/+ mice and 8.3 μg/g for *op*/*op* mice (difference of 2.7-fold). The +/+ and *op*/*op* mice that survived beyond 50 h gradually recovered, began to gain weight, and appeared normal for the next 6 weeks. These results confirmed earlier preliminary data obtained in a study using fewer mice (18).

However, when the *op*/*op* mice were pretreated with recombinant human CSF-1 (10^5 U per mouse per day administered i.p. for 10 days), their response to endotoxin was dramatically enhanced, and five of five mice died after administration of 2 μg of LPS per g. This observation provided additional evidence of the role of CSF-1-dependent macrophages in mediating LPS toxicity. Combined, these data suggest that the *op*/*op* mice are significantly more resistant to endotoxin than control mice and that the increased susceptibility to endotoxin was not responsible for the increased susceptibility of mutant mice to *E. coli* peritonitis. These data are compatible with our earlier observation that the *op*/*op* mice release significantly less TNF-α and IL-1α (18, 21) in response to LPS. Both TNF-α and IL-1α have been shown to be mediators of LPS-induced mortality (3, 13).

Increased susceptibility of the *op*/*op* control mice to recombinant human TNF-α and IL-1α. Because of the scarcity of mutant mice, the question of susceptibility could not be addressed in a completely independent way. However, in experiments attempting to evaluate the role of TNF-α and IL-1α in stimulation of bone resorption in the *op*/*op* mice, marked differences in survival of similarly treated *op*/*op* and control mice were observed. While 6 of 10 +/+ mice survived the administration of 1 μg of rhTNF-α per g of body weight, none of 10 *op*/*op* mice survived this dose ($P < 0.05$); only 2 of 5 *op*/*op* mice survived administration of 1/10 this dose, i.e., 0.1 μg/g of body weight, whereas five of five +/+ mice survived administration of the lower dose of TNF-α. Mice that died did so during the first 2 days, and the remaining mice survived to day 7, when they were sacrificed and analyzed for bone resorption. In a separate experiment, groups of *op*/*op* and control mice were treated with IL-1α. Only 2 of 10 *op*/*op* mice that had received IL-1α on 3 consecutive days survived 7 days. Treated mice died on day 4 or 5 after IL-1α administration had begun. In contrast, 10 of 10 IL-1α-treated weight-matched normal mice survived to day 7 ($P < 0.01$), when all surviving mice were sacrificed for the evaluation of bone resorption.

Secondary challenge of the *op*/*op* and control mice with *E. coli*. Five of the *op*/*op* and eight control mice that survived the infection with 10^2 or 10^3 *E. coli* and were not sacrificed for other evaluation were inoculated with 10^6 *E. coli* along with feces 2 months after primary infection. This time was chosen because in preliminary experiments macrophages induced in the peritoneal cavities of *op*/*op* mice were found to disappear after approximately 1 month (data not shown) without further stimulation. Three of five *op*/*op* and seven of eight control mice survived this infection with bacteria at a dose clearly lethal to both types of mice on primary challenge. The course of disease was also more smouldering than during primary infection, and mice that died did so on days 5 and 6 postinfection. Although derived from a relatively small number of animals, these data clearly suggest that the *op*/*op* mice are capable of an efficient secondary response to *E. coli* fecal peritonitis.

DISCUSSION

These studies have shown that *op*/*op* mice, which are nearly devoid of peritoneal macrophages as well as blood monocytes and possess profound macrophage deficiencies in other organs, are more susceptible than normal mice to fecal peritonitis caused by nonpathogenic *E. coli*, but that the increase in susceptibility is only about 30-fold.

Since death resulting from this infection is rapid, occurring within 24 h postinfection, and associated with a clinical course resembling that of postendotoxin shock, the difference in mortality could be due either to the difference in the kinetics of cells recruited to control local infection, leading to a significantly decreased rate of elimination of bacteria and subsequent increased release of endotoxin in the infected *op*/*op* mice, or to the difference in sensitivity to endotoxin between mutant and control animals.

More detailed analysis of cellular changes associated with this infection in the peritoneal cavity has revealed that most likely this increase in susceptibility is due to delayed and reduced recruitment of neutrophils to peritoneal cavities of infected mice. This view is supported by the following arguments: (i) changes in the numbers of other cells do not occur at a time which is critical for survival; (ii) both the time of appearance and the magnitude of the increase in the number of neutrophils suggest their direct involvement in fighting infection at the time critical for survival; (iii) neutrophils are the only professional phagocytes that are available in large numbers at the site of infection at that time both in control and in mutant infected mice; and (iv) pretreatment of *op*/*op* mice with CSF-1 corrects the delayed neutrophil response to challenge with *E. coli* and improves their survival.

The increased resistance of the *op*/*op* mice to LPS evident without galactosamine pretreatment may serve as additional support for the conclusion that the difference in neutrophil recruitment is responsible for increased sensitivity of *op*/*op* mice to *E. coli* peritonitis, as it rules out one alternative explanation of the increased mortality during fecal peritonitis, i.e., increased susceptibility to LPS. However, it is quite likely that LPS eventually contributes to the mortality of the *op*/*op* mice during fecal peritonitis. The possible scenario would begin when injected bacteria encounter the absence of resident macrophages and reduced influx of neutrophils. In this situation, they would multiply better and release more LPS than in control mice. This increased release of LPS may induce still sub-

stantial amounts of both TNF-α and IL-1α from alternative sources. In turn, these mediators acting in the situation of increased susceptibility of the *op*/*op* mice to these molecules might still contribute to their increased mortality. Such a scenario is compatible not only with the data obtained in this study but also with evidence gathered from the literature regarding postendotoxin shock and the roles of TNF-α and IL-1α in this situation (2, 17, 19).

The deficiency in the recruitment of neutrophils to the peritoneal cavities of *op*/*op* mice infected with *E. coli* may be partly explained by the deficiency in the release of granulocyte colony-stimulating factor observed after injection of LPS (22). Other putative mediators of the induced influx of neutrophils to the peritoneal cavity could include IL-8 (10) and many other macrophage products, such as transforming growth factor β, IL-α, TNF-α, platelet-activating factor, leukotriene B_4, IL-1β (2, 17), and macrophage inflammatory protein 1 (10). The deficiency of macrophage-derived neutrophil-attracting cytokines could be explained by macrophage deficiency in the peritoneal cavities of *op*/*op* mice. Consequently, these data clearly suggest a role for resident tissue macrophages.

The CSF-1-dependent macrophages absent in the *op*/*op* mice do not seem to be required to mount an effective secondary response. However, the present data clearly demonstrate that in the presence of a secondary immune response, the deficiency in primary response does not seem to play a critical biological role, since even such significant local macrophage deficiency as in the *op*/*op* mice did not compromise very significantly their survival, provided that they were preimmunized.

In conclusion, the present data demonstrate that the CSF-1-dependent resident macrophage subpopulation in the mouse plays a role in the primary response to acute bacterial infection, but this role is mainly regulatory and dependent on effective recruitment of other cells such as neutrophils. This study also demonstrates the usefulness of the *op*/*op* mouse as a model for investigation of the role of macrophages in the immune response to various infections.

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