Elimination of Resident Macrophages from the Livers and Spleens of Immune Mice Impairs Acquired Resistance against a Secondary *Listeria monocytogenes* Infection

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During a secondary *Listeria monocytogenes* infection in mice, the bacteria are eliminated more rapidly from the liver and spleen than during a primary infection. This acquired resistance against a secondary infection is dependent on T lymphocytes, which induce enhanced elimination of bacteria via stimulation of effector cells such as neutrophils, resident macrophages, exudate macrophages, and hepatocytes. The aim of the present study was to determine the role of the resident macrophages in acquired resistance against a secondary *L. monocytogenes* infection in mice. Mice which had recovered from a sublethal primary infection with 0.1 LD50 of *L. monocytogenes* intravenously (i.v.), i.e., immune mice, received a challenge of 1 LD50 of *L. monocytogenes* i.v. to induce a secondary infection. At 2 days prior to challenge, immune mice were given an i.v. injection of liposomes containing dichloromethylene-diphosphonate (L-Cl2MDP) to selectively eliminate resident macrophages from the liver and spleen. Control immune mice received either phosphate-buffered saline (PBS) or liposomes containing PBS (L-PBS). Treatment of mice with L-Cl2MDP effectively eliminated resident macrophages from the liver and spleen but did not affect the number of granulocytes, monocytes, or lymphocytes in peripheral blood or their migration to a site of inflammation. Phagocytosis and killing of *L. monocytogenes* by peritoneal exudate cells elicited with heat-killed *L. monocytogenes* were similar in all groups of immune mice. On day 3 of a secondary infection, the number of *L. monocytogenes* organisms in the livers and spleens of L-Cl2MDP-treated immune mice was 4 log10 units higher than in immune mice treated with PBS or L-PBS. The concentration of reactive nitrogen intermediates in plasma, a measure of the severity of infection, was 70-fold higher for L-Cl2MDP-treated immune mice than for PBS- or L-PBS-treated immune mice. Treatment with L-Cl2MDP significantly increased the number of inflammatory foci in the liver and spleen, decreased their size, and affected their structure. From these results, we conclude that resident macrophages are required for the expression of acquired resistance against a secondary *L. monocytogenes* infection in mice.

Mice that survive a primary *Listeria monocytogenes* infection, i.e., immune mice, are able to eliminate the bacteria much faster and more efficiently from their organs during a secondary infection than nonimmune mice with a primary infection (21). This acquired resistance against infection is mediated by two groups of cells: inducers of acquired resistance and effectors of acquired resistance. Mackeness identified T lymphocytes as the major inducers of acquired resistance by the observation that adoptive transfer of spleen cells from *Listeria*-immune mice to naive mice protected the latter against a *L. monocytogenes* infection (22). CD8+ T lymphocytes were found to be the most effective inducers of acquired resistance, whereas CD4+ T lymphocytes were thought to be involved in the delayed-type hypersensitivity reaction and granuloma formation (14, 23). However, recent studies with major histocompatibility complex type I (MHC1) and MHCII knockout mice, which are devoid of functional CD4+ and CD8+ αβ T lymphocytes, respectively, revealed that both T-lymphocyte subsets are necessary for the induction of acquired resistance against a secondary *L. monocytogenes* infection (18).

Efficient elimination of bacteria from the organs during a secondary infection is dependent on effector cells. Irradiation of naive mice before an adoptive transfer of spleen cells from *Listeria*-immune mice abrogated the protection against a *L. monocytogenes* infection, demonstrating that bone marrow-derived effector cells are necessary for the expression of acquired resistance (13). Treatment of naive mice with dextran sulfate 500, a compound which affects the phagocytic capacity of mononuclear phagocytes, also inhibited the expression of acquired resistance against infection after adoptive transfer, confirming that the bone marrow-derived effector cells are the mononuclear phagocytes (12). Recently, it has been demonstrated that neutrophils can be selectively depleted with a monoclonal antibody and that these cells are also required for the elimination of bacteria during a secondary *L. monocytogenes* infection (5, 6, 29). Since irradiation and dextran sulfate affect various cell types, the role of mononuclear phagocytes, in particular the resident macrophages and the exudate macrophages, in the elimination of bacteria during a secondary infection in mice should be reevaluated. Recently, a method has been described which allows selective depletion of resident macrophages without affecting monocytes or granulocytes (39). The aim of the present study is to determine the role of the resident macrophages in acquired resistance against a secondary *L. monocytogenes* infection in mice.

**MATERIALS AND METHODS**

Mice. Female, specific-pathogen-free CBA/J mice, aged 6 to 8 weeks, were purchased from IFFA Credo (Saint Germaine-sur-l’Aberle, France) and given dry food (Hope Farms, Woerden, The Netherlands) and tap water ad libitum.
Bacteria. L. monocytogenes EGD was kept virulent by repeated passage through CBA/J mice and stored on blood agar plates at 4°C. The bacteria were cultured in tryptose phosphate broth for 18 h at 37°C, collected by centrifugation (10 min at 900 × g), washed in phosphate-buffered saline (pH 7.4) (PBS), and resuspended in pyrogen-free saline.

For several experiments the bacteria were preopsonized by incubation of 2 × 10^8 bacteria/ml with 20% (vol/vol) normal mouse serum for 30 min at 37°C under rotation (4 rpm) followed by centrifugation (10 min at 900 × g). After washing twice with Hank’s balanced salt solution supplemented with 0.1% gelatin and 10 mM HEPES (gelatin-HBSS), the bacteria were resuspended in gelatin-HBSS to a concentration of 10^7/ml.

Monoclonal antibodies. The following monoclonal antibodies (MAb) were used: M1/70, a rat immunoglobulin G2a (IgG2a) antibody that recognizes the marginal metallophilic macrophages in the mouse spleen (16); M2/1, a rat IgG2k antibody used as a pan-macrophage marker (17); ERTR-9, a rat IgM antibody that recognizes the splenic marginal-zone macrophages (41); and M1/70, a rat IgG2k anti-CR3 α chain (CD11b, MAC-1 α chain) which recognizes monocyte-derived exudate macrophages (34). For immunohistochemistry, the MAb were used in the form of tissue culture supernatants.

Liposome preparation. Multilamellar liposomes containing either dichloromethylene diphosphonate (Cl2MDP; a gift from Boehringer Mannheim GmbH, Mannheim, Germany) or PBS were prepared as previously described (39). Briefly, 75 mg of phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and 11 mg of cholesterol (Sigma Chemical Co., St. Louis, Mo.) were dissolved in chloroform, evaporated by rotation under vacuum at 37°C, dispersed by mixing with 10 ml of PBS containing 2.5 g of Cl2MDP for 10 min, placed at room temperature (RT) for 2 h, sonicated for 3 min at RT in a water bath sonicator, and placed at RT for 2 h. The resulting liposomes containing Cl2MDP (L-Cl2MDP) were washed twice in PBS by centrifugation at 100,000 × g for 30 min to remove nonencapsulated Cl2MDP. PBS-containing liposomes (L-PBS) were prepared by the same procedure, except that the phosphatidylcholine-cholesterol mixture was dispersed in 10 ml of PBS without Cl2MDP. The L-Cl2MDP and L-PBS were each resuspended in 4 ml of PBS, and 0.2 ml of either preparation was injected intravenously (i.v.) per mouse.

Induction of primary or secondary L. monocytogenes infection in mice. A secondary infection was induced in mice by first injecting 5 × 10^8 L. monocytogenes (0.1 LD50% lethal dose [LD50]) i.v. (36). At 19 days later, the mice, considered immune, received 0.2 ml of L-Cl2MDP, L-PBS, or PBS i.v., and 2 days later, they were given 5 × 10^7 L. monocytogenes i.v. (1 LD50). A primary L. monocytogenes infection was induced in naive mice by injecting 1 LD50 of L. monocytogenes i.v. One day later primary or secondary infection, blood samples were taken from the mice by heart puncture, collected in a heparinized tube, and stored as plasma at −20°C until use. The liver and spleen were removed and homogenized with a tissue homogenizer (type X-1020; Ystral GmbH, Döttingen, Germany). Serial 10-fold dilutions of the organ suspensions were plated onto blood-agar plates and incubated for 24 h at 37°C. The number of colonies was used to calculate the number of viable L. monocytogenes cells per organ.

Analysis of blood leukocytes and PEC. Immune mice were given 0.2 ml of L-Cl2MDP, L-PBS, or PBS i.v., and after 2 days blood samples were taken by puncture of the retroorbital plexus and collected in a heparinized tube. Total blood leukocytes were counted with a Coulter Counter (model 2F; Coulter Electronics Ltd., Luton, England). The percentages of blood granulocytes, monocytes, and lymphocytes were determined by differential counting of blood smears stained with Giemsa and May-Grunwald. To obtain peritoneal exudate cells (PEC), immune mice were first treated with L-Cl2MDP, L-PBS, or PBS i.v. and after 2 days given an ip injection of 5 × 10^7 heat-killed (70°C for 10 min) L. monocytogenes cells. Two days later, PEC were collected by peritoneal lavage with 2 ml of ice-cold PBS containing 50 U of heparin per ml, as previously described (37). The composition of the cell suspension was determined by analysis of Giemsa-stained cytospin preparations. The remaining cells were used for in vitro phagocytosis and killing of L. monocytogenes.

In vitro phagocytosis and killing of L. monocytogenes by inflammatory leukocytes. In vitro phagocytosis and killing were performed as previously described (19). In short, a suspension containing 10^7 PEC/ml and 10^8 preopsonized bacteria/ml was incubated for 15 min at 37°C under rotation (4 rpm). After phagocytosis, the PEC were centrifuged at 240 × g for 4 min at 4°C and washed three times with ice-cold gelatin-HBSS to remove bacteria that were not cell associated. The PEC were resuspended at the original concentration in gelatin-HBSS containing 10% (vol/vol) normal mouse serum. Cytosin preparations with 10^4 PEC were made to determine the percentage of cells with cell-associated bacteria. The remaining PEC were divided into three aliquots that were incubated for 0, 30, and 60 min at 37°C under rotation (4 rpm). After incubation, the PEC were lysed by addition of H2O containing 0.01% (vol/vol) bovine serum albumin (BSA) under vigorous mixing for 1 min. Serial 10-fold dilutions were plated onto blood-agar plates, and the number of viable bacteria, i.e., intracellular and cell-associated bacteria, was determined microbiologically. The intracellular killing was expressed by a decrease in the percentage of bacteria at 30 and 60 min relative to the beginning of the assay at 0 min. At the end of the assay, the viability of the PEC as indicated by trypan blue exclusion exceeded 95%.

Measurement of RNI. The concentration of nitrite (NO2−) and nitrate (NO3−), which are stable metabolites of nitric oxide (NO), was measured in the plasma of infected mice. A plasma sample was diluted twofold with deionized H2O and deproitinized with 30% zinc sulfate; NO2− in the sample was reduced to NO3− by incubation with spongy monkey (11); the concentration of NO3− was determined with Griess reagent (25) and referred to as reactive nitrogen intermediates (RNI).

FIG. 1. Number of L. monocytogenes cells in the livers and spleens and concentration of RNI in plasma of L-Cl2MDP-treated immune mice with a secondary L. monocytogenes infection. Mice were immunized with 0.1 LD50 of L. monocytogenes i.v. (III); nonimmunized mice received saline (C). After 19 days, the immunized mice were given 0.2 ml of L-Cl2MDP, L-PBS, or PBS, and 2 days later all the mice received 1 LD50 of L. monocytogenes i.v. On day 3 of infection, the number of viable L. monocytogenes cells in the livers (A) and spleens (B) and the concentration of RNI in plasma (C) were assessed. Data are means ± SD (n = 8). Two of the eight Cl2MDP-treated immune mice had died on day 3 of infection.
RESULTS

Number of L. monocytogenes cells in liver and spleen and concentration of RNI in plasma of L-Cl2MDP-treated immune mice with a secondary infection. On day 3 of a secondary infection, the number of L. monocytogenes in the livers of immune mice treated with L-Cl2MDP was significantly larger (mean difference, log10 5.03 ± 1.25) than that of PBS- or L-PBS-treated immune mice and was also larger (mean difference, log10 3.69 ± 1.36) than that of nonimmune mice with a primary infection (Fig. 1A). The number of bacteria recovered from the spleens of immune mice treated with L-Cl2MDP was significantly increased (mean difference, log10 4.23 ± 1.14) in comparison with that of PBS- or L-PBS-treated mice on day 3 of secondary infection and was slightly (mean difference, log10 1.32 ± 0.32) larger than that of nonimmune mice with a primary infection (Fig. 1B). On day 3 of a secondary infection, two of eight L-Cl2MDP-treated immune mice had died and the remaining six mice were cachectic, characterized by extensive weight loss, decreased body temperature, and immobility. All PBS- and L-PBS-treated immune mice and mice with a primary infection survived and were otherwise healthy.

Since we have demonstrated that the concentration of RNI in plasma can be considered a parameter for the severity of infection (33), the concentration of RNI in plasma was determined in PBS-, L-PBS-, and L-Cl2MDP-treated immune mice with a secondary infection and in mice with a primary infection. On day 3 of infection, the concentration of RNI in the plasma of immune mice treated with L-Cl2MDP was 70-fold higher than that in the plasma of immune mice treated with PBS or L-PBS and of nonimmune mice with a primary infection (Fig. 1C).

Peripheral blood leukocytes in L-Cl2MDP-treated immune mice. To confirm that L-Cl2MDP treatment did not affect the number of peripheral blood leukocytes in immune mice, total blood leukocyte counts and differential counts were performed before a secondary infection with L. monocytogenes. Two days after L-Cl2MDP treatment, the total number of leukocytes in the blood of L-Cl2MDP-treated immune mice was not different from that in the blood of L-PBS-treated immune mice and nonimmune mice but was slightly larger (P < 0.05) than that in the blood of PBS-treated immune mice (Table 1). This increase in the number of leukocytes due to i.v. injection of liposomes has also been described by others (28, 31).

The percentages of monocytes, lymphocytes, and granulocytes in the blood of L-Cl2MDP-treated immune mice were comparable to those in the blood of nonimmune and PBS-treated immune mice (P > 0.05) (Table 1). Treatment with L-PBS induced a slight increase (P < 0.05) in the percentage of blood monocytes in immune mice (Table 1).

Migration and functional activity of peritoneal exudate cells from L-Cl2MDP-treated immune mice. To ensure that L-Cl2MDP does not affect the migration of leukocytes from the blood to the site of inflammation, the migration of leukocytes to the peritoneal cavity in response to an intraperitoneal stimulus was studied. Two days after injection of heat-killed L. monocytogenes into the peritoneal cavity, the total number of PEC from L-Cl2MDP-treated immune mice did not significantly differ from the total number of PEC from nonimmune and PBS- and L-PBS-treated immune mice (Table 2). The percentages of macrophages and granulocytes recovered from the peritoneal cavity of immune mice were higher than those from nonimmune mice (Table 2), but within the groups of immune mice, the percentages of macrophages, lymphocytes, and granulocytes were similar (Table 2).

To determine whether i.v. treatment with L-Cl2MDP affected the functional activity of migrating leukocytes, the phagocytosis and killing of L. monocytogenes by the PEC was
investigated. After in vitro phagocytosis of live *L. monocytogenes* for 15 min, the percentage of PEC with cell-associated bacteria did not differ from that in PBS-, L-PBS-, and L-Cl2MDP-treated immune mice (P > 0.05) (Table 3). Treatment with L-Cl2MDP i.v. did not alter the killing of *L. monocytogenes* by PEC from the immune mice (P > 0.05) (Table 3).

**Effect of L-Cl2MDP treatment on hepatocytes.** Hepatocytes are a major site for replication of *L. monocytogenes* in the liver (10). To exclude that the effect of L-Cl2MDP treatment on bacterial proliferation in the liver was caused by toxic effects of this compound on hepatocytes, the levels of the liver enzymes ALT and AST in the serum of L-Cl2MDP-treated uninfected mice (n = 5) were measured. The concentration of ALT in the serum of PBS-treated mice (37.1 ± 12.7 U/liter [mean ± standard error of the mean, SEM]) was not significantly different (P > 0.05) from that in the serum of L-Cl2MDP-treated mice (55.1 ± 5.72 U/liter). Furthermore, the concentrations of AST in the serum of PBS-treated mice (360.7 ± 241.2 U/liter) and L-Cl2MDP-treated mice (349.2 ± 30.7 U/liter) were similar (P > 0.05). These data demonstrate that L-Cl2MDP treatment does not induce excessive liver damage.

**Histology.** In the spleens of immune mice treated with L-Cl2MDP, almost no acid phosphatase-positive cells, i.e., resident macrophages, could be detected on day 3 of a secondary infection except for a few positive cells in the white pulp (Fig. 2b). In the spleens of PBS-treated immune mice, acid phosphatase-positive cells were abundant on day 3 of a secondary infection (Fig. 2a). No marginal-zone macrophages (Fig. 2d) or marginal metallophilic macrophages (data not shown) could be detected in the spleens of L-Cl2MDP-treated mice with the MAb ERTR-9 (41) and MOMA-1 (16), respectively, whereas many positive cells were present in the spleens of PBS-treated immune mice (Fig. 2c). In the livers of L-Cl2MDP-treated immune mice, no acid phosphatase-positive Kupffer cells could be found on day 3 of a secondary infection, whereas in the livers of PBS-treated immune mice, positively staining Kupffer cells were abundant (data not shown). Immunoperoxidase staining with MAb M1/70 (34) on day 3 of a secondary infection showed many exudate macrophages in inflammatory foci in the livers (Fig. 2e and f) and spleens (data not shown) of PBS- and L-Cl2MDP-treated immune mice. The number of inflammatory foci per square millimeter of liver section was significantly increased in L-Cl2MDP-treated immune mice in comparison to PBS- and PBS-treated immune mice with a secondary infection (Fig. 3). In PBS-treated immune mice, the inflammatory foci were large and consisted of a center with densely packed exudate macrophages surrounded by a layer of necrotic cells, granulocytes, and exudate macrophages (Fig. 2e). In L-Cl2MDP-treated immune mice, the inflammatory foci were small, did not have a well-defined structure, and consisted mainly of exudate macrophages (Fig. 2f). The granulocytes in the livers (Fig. 2e) and spleens (data not shown) of PBS-treated immune mice were confined to well-structured inflammatory foci, whereas the granulocytes in L-Cl2MDP-treated immune mice were scattered throughout the liver (Fig. 2f). The number of inflammatory foci in the livers of L-PBS-treated immune mice was similar to that in PBS-treated immune mice but approximately twofold lower than in mice with a primary infection. These results confirm that the immune mice possess acquired resistance against a secondary infection (20).

**Number of *L. monocytogenes* cells in the livers and spleens and concentration of RNI in plasma of Cl2MDP-treated immune mice given an injection of TNF during a secondary infection.** Since TNF is essential for acquired resistance against a secondary infection (32) and activated macrophages secrete TNF, the effect of administration of TNF to L-Cl2MDP-treated immune mice that are devoid of resident macrophages was assessed. Intravenous injection of TNF 1 h prior to a secondary infection tended to decrease the number of *L. monocytogenes* cells in the livers (P > 0.05) and spleens (P > 0.05) and the number of inflammatory foci in the livers of L-Cl2MDP-treated immune mice (2.41 ± 0.25) (P > 0.05) in comparison with L-Cl2MDP-treated immune mice that received saline (2.73 ± 0.49). However, the concentration of RNI in plasma was significantly decreased (P < 0.05) in these mice.

### Table 2. Effect of treatment with L-Cl2MDP on the migration of leukocytes to the peritoneal cavity

<table>
<thead>
<tr>
<th>Status of mouse</th>
<th>Total leukocytesa (10⁶)</th>
<th>Differential count (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macrophages</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Naive</td>
<td>4.1 ± 2.0</td>
<td>44.3 ± 13.5</td>
</tr>
<tr>
<td>Nonimmune</td>
<td>6.8 ± 1.0</td>
<td>61.5 ± 16.3</td>
</tr>
<tr>
<td>Immune + PBS</td>
<td>8.2 ± 2.0</td>
<td>74.5 ± 9.0</td>
</tr>
<tr>
<td>Immune + L-PBS</td>
<td>8.2 ± 2.6</td>
<td>75.6 ± 5.8</td>
</tr>
<tr>
<td>Immune + L-Cl2MDP</td>
<td>5.4 ± 0.7</td>
<td>76.0 ± 4.7</td>
</tr>
</tbody>
</table>

a Mice were immunized with 0.1 LD₅₀ of *L. monocytogenes*; nonimmunized mice received saline. After 19 days, the immunized mice were given 0.2 ml of L-Cl2MDP, L-PBS, or PBS, and 2 days later, all mice received an intraperitoneal injection of 5 × 10⁶ heat-killed *L. monocytogenes*. After 2 days, peritoneal cells were harvested and counted.

b Values are means ± SD for four mice.
c P < 0.05 in comparison with immune mice.

d P < 0.05 in comparison with immune mice.

### Table 3. Effect of treatment with L-Cl2MDP on the phagocytosis of intracellular bacteria in the liver and spleen

<table>
<thead>
<tr>
<th>Status of mouse</th>
<th>% of PEC with cell-associated bacteria</th>
<th>% of viable intracellular and cell-associated bacteria at time (min):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Immune + PBS</td>
<td>18.0 ± 3.9</td>
<td>100</td>
</tr>
<tr>
<td>Immune + L-PBS</td>
<td>17.5 ± 3.7</td>
<td>100</td>
</tr>
<tr>
<td>Immune + L-Cl2MDP</td>
<td>21.5 ± 1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

a Mice were immunized with 0.1 LD₅₀ of *L. monocytogenes* and after 19 days were given 0.2 ml of L-Cl2MDP, L-PBS, or PBS i.v. Two days later, a sterile peritonitis was induced by intraperitoneal injection of 5 × 10⁹ heat-killed *L. monocytogenes*. PEC were recovered 2 days thereafter and incubated with preopsonized viable *L. monocytogenes* for 15 min at 37°C under rotation. Values are means ± SD for four mice.

c The percentage of PEC with cell-associated bacteria was assessed microscopically.

d After incubation for 0, 30, and 60 min at 37°C under rotation, the cells were lysed and the number of live bacteria, i.e., viable intracellular and cell-associated bacteria, was assessed. The killing was reflected as the decrease in the percentage of bacteria at 30 and 60 min relative to that at 0 min.
L-Cl2MDP-treated immune mice (Fig. 4). Injection of TNF 24 h after challenge did not affect the number of bacteria in the livers and spleens or the concentration of RNI in the plasma of L-Cl2MDP-treated immune mice (Fig. 4).

DISCUSSION

From the above results, it can be concluded that resident macrophages are required for the expression of acquired resistance during a secondary L. monocytogenes infection in immune mice. This conclusion is supported by the observation that during a secondary infection in mice depleted of resident macrophages, the number of L. monocytogenes cells in the livers and spleens was significantly higher than that in control mice. Furthermore, the concentration of RNI in the plasma of these mice, which is considered a measure for the severity of Listeria infection (33), was dramatically increased in comparison with control mice. In addition, in the livers and spleens of these mice the inflammatory foci increased in number and drastically decreased in size in comparison with control mice.

In this study, i.v. treatment with liposome-encapsulated Cl2MDP was used to selectively deplete resident macrophages from the livers and spleens of immune mice. The efficacy of the method was assessed by histological evaluation of the livers and spleens, which confirmed that all resident macrophages were eliminated except for a few positively stained macrophages in the white pulp of the spleen, as has been found previously (28, 31, 39). Treatment with L-Cl2MDP was not hepatotoxic and did not affect the number of monocytes, lymphocytes, and granulocytes in the circulation.

Despite the presence of granulocytes and monocyte-derived exudate macrophages in the livers and spleens of macrophage-depleted immune mice, the bacteria were not eliminated and even increased in number. Are the phagocytes in these organs impaired in their function? It is unlikely that L-Cl2MDP taken up by resident macrophages is released as free Cl2MDP upon the death of these cells and affects the function of granulocytes and exudate macrophages, scattered through the liver as observed in L-Cl2MDP-treated immune mice on day 3 of infection.

FIG. 3. Number of inflammatory foci per square millimeter of tissue section in the livers of L-Cl2MDP-treated mice with a secondary infection. Mice were immunized with 0.1 LD50 of L. monocytogenes i.v. (II); nonimmunized mice received saline (II). After 19 days, the immunized mice were given 0.2 ml of L-Cl2MDP, L-PBS, or PBS, and 2 days later, all mice received 1 LD50 of L. monocytogenes i.v. On day 3 of infection, the liver was removed and cryosectioned. The sections were stained for CR3-positive macrophages. The number of granulomatous lesions was assessed microscopically. Data are means ± SD (n = 4).

Another important characteristic of resident macrophages,
infection (24). In addition, we have demonstrated that neutralization of TNF during a secondary infection dramatically increases the number of *L. monocytogenes* cells in the liver and spleen (32). Since the course of a secondary infection in the livers and spleens of anti-TNF-treated immune mice is similar to that in L-Cl2MDP-treated immune mice, we supposed that administration of TNF to L-Cl2MDP-treated immune mice could restore their resistance against an infection. Our findings demonstrate that although treatment with TNF prior to a secondary infection only slightly decreased the number of bacteria in the liver and spleen and the number of inflammatory foci in the liver, it significantly reduced the concentration of RNI in plasma from immune mice depleted of resident macrophages. The latter observation indicates that treatment with TNF somewhat attenuates the *Listeria* infection in macrophage-depleted immune mice. Whether higher concentrations of TNF, longer treatment with this cytokine, or treatment with a mixture of cytokines can substitute for the paracrine function of resident macrophages has not yet been elucidated.

In sum, our findings demonstrate that besides neutrophils and exudate macrophages, resident macrophages are required for the expression of acquired resistance against a secondary *L. monocytogenes* infection in mice.

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