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Marginal Zone Macrophages and Immune Responses Against Viruses

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The effective establishment of antiviral protection requires a coordinated interplay between the innate and adaptive immune system. Using osteopetrotic (op−/−) mice, this study investigated the influence of marginal zone macrophages in controlling and initiating a protective immune response against a cytopathic vs a non- or low-cytopathic virus. Despite the generation of potent adaptive immune responses, antiviral protection against cytopathic vesicular stomatitis virus critically depended on the presence of marginal zone macrophages. Infection with low doses (100 PFU) of non- or low-cytopathic lymphocytic choriomeningitis virus was usually cleared and usually resulted in a carrier state in the majority of mice. This shows that the early innate immune system provides an important preparatory phase to the adaptive immune system and is particularly important for antiviral protection. The Journal of Immunology, 2002, 169: 1453-1458.

For efficient immunity, the different components of the immune system have to cooperate. The interplay among lymphokines, chemokines, and cellular and humoral immunity has been studied in numerous models of natural and targeted mutations (1-3). However, collaboration between the innate and the adaptive immune system is not well explored because defects affecting the innate immune system, particularly macrophages or neutrophils, are often embryonically lethal or severely immunocompromise the host, leading to death soon after birth. Osteopetrotic (op) mice carry a natural point mutation in the M-CSF gene (4-7) and fail to develop marginal zone macrophages (MZM) in the spleen and the corresponding sinusoidal macrophages in the lymph nodes (8, 9); these mice are severely deficient in functional osteoclasts and therefore form inadequate bone marrow cavities. Despite these defects, op−/− mice develop normal in vivo phagocytic functions but have impaired release of TNF and G-CSF (10). Although B cell lineage precursors are present in the bone marrow of these mice, B cell lymphopoiesis in adult op−/− mice is observed only in the liver (11). Despite this, op−/− mice demonstrate normal B cell responses in vivo (10, 12). Normal MLRs in vitro indicated that T cells from op−/− mice are functional (12).

It has been observed that macrophage depletion reduces the host capacity to control viral infections (13, 14). Depletion of MZM can be experimentally induced by the administration of clodronate encapsulated in liposomes (15-17). Using this experimental approach, it has been shown that such mice fail to control a systemic lymphocytic choriomeningitis virus (LCMV) infection (18). However, long-term studies are difficult to carry out in MZM-depleted mice because the MZM are replenished and because repeated treatment with clodronate liposomes results in a high mortality rate with severe side effects resulting in noninterpretable results. Nevertheless, studies in MZM-depleted mice and in op−/− mice revealed that MZM play a role in controlling Listeria monocytogenes infections (19, 20). Additionally, earlier studies had shown that LCMV-infected MZM were destroyed by virus-specific CTLs and caused systemic immunosuppression (21). Non- or low-cytopathic viruses such as LCMV differ from cytopathic viruses such as vesicular stomatitis virus (VSV) in that the former are usually eliminated by CTLs, whereas neutralizing Abs induced during the course of the primary immune response usually control the latter. Common to both viruses is that they are initially recruited to lymphoid organs by natural Abs, where they are thought to be removed by MZM to reduce dissemination of virus to vital organs and at the same time enhance induction of an immune response (22-24). The present study used op−/− mice to evaluate the contribution of MZM to the elimination of a non- or low-cytopathic virus vs a cytopathic virus and to investigate the effect of impaired removal of infectious virus on antiviral immunity and disease manifestation.

Materials and Methods
Mice and viruses

C57BL/6 mice were obtained from the breeding colony of the Institut für Laborierkunde (Faculty of Veterinary Medicine, University Zurich-Irchel, Zurich, Switzerland). Experiments were conducted with age- and sex-matched animals kept under specific pathogen-free conditions. Heterozygous op/+ mice were obtained from The Jackson Laboratory (Bar Harbor, ME), embryo-derived, back-crossed onto C57BL/6 for two generations, and kept under specific pathogen-free conditions according to institutional guidelines. Heterozygous breeding pairs were identified by PCR as previously described (5). Inspecting them for the lack of incisors identified homozygous op−/− offspring. They were fed powdered mouse food and water supplemented with 5% glucose. Heterozygous and wild-type littermates were separated and sacrificed as early as possible from op−/− littermates to increase the survival rate of op−/− mice and to obtain a sufficient number of homozygotes. For this reason, i.e., lack of littermates, all experiments were conducted with age-matched (date of birth within the
supernatant was harvested and radioactivity determined in a gamma counter, and the percentage of specific release was calculated.

Secondary in vitro restimulation

A total of $5 \times 10^5$ spleen cells and $2 \times 10^5$ LCMV-infected peritoneal exudate macrophages were cocultured in 24-well tissue culture plates in a total volume of 2 ml IMDM supplemented with 10% FCS, penicillin/streptomycin, glutamine, and $5 \times 10^{-3}$ M 2-ME. Macrophages were obtained by injecting mice i.p. with 1 ml thioglycolate on day −6 and with 200 PFU LCMV on day −4. After 5 days, 1 ml medium was removed and 100 µl of these standard cultures were diluted in duplicate (four steps, 3-fold dilutions) in MEM supplemented with 2% FCS in 96-well round-bottom plates and specific cytotoxicity was determined as described above.

Immunohistochemistry

Freshly removed organs were immersed in HBSS and snap-frozen in liquid nitrogen. Tissue sections of 5-µm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed with 4% formalin in PBS, and infectious foci were detected by immunohistochemistry.

Cytotoxicity assay

The cytolytic activity of spleen cells was tested in a $^{3}H$Cr release assay as described previously (29). Briefly, EL-4 target cells were coated with LCMV-derived peptide gp33–41 (30) at concentrations of $10^{-6}$ M. A total of $10^5$ target cells were incubated in 96-well round-bottom plates with serial 3-fold dilutions of spleen effector cells starting at an E:T ratio of 70:1 in a final volume of 200 µl. After 5 h of incubation at 37°C, 70 µl of supernatant was harvested and radioactivity determined in a gamma counter, and the percentage of specific release was calculated.

FIGURE 1. $op^{-/-}$ mice fail to develop a footpad swelling reaction. $op^{-/-}$ (op) and C57BL/6 (B6) mice were injected into the hind footpad with 100 PFU LCMV and the footpad swelling reaction was monitored daily for the indicated time period. Each line represents one animal. Some mice were sacrificed for other experiments (discontinued lines). Data from two independent experiments are shown.
Footpad swelling reaction

Mice were injected into the hind footpad with 100 PFU LCMV WE in 30 μl of MEM supplemented with 2% FCS. The footpad thickness was measured with a spring-loaded caliper (Kroeplein; Schluchtern, Hessen, Germany). Values were taken as the mean of both hind footpads (29, 33).

Results

op<sup>−/−</sup> mice fail to generate a primary footpad swelling reaction

To assess the capacity of op<sup>−/−</sup> mice to mount an immune response against non- or low-cytotoxicity LCMV, mice were infected via the footpad (i.f.) with LCMV. After footpad infection, immunocompetent mice develop a footpad swelling reaction due to a biphasic CD8<sup>+</sup> and CD4<sup>+</sup> T cell response (29, 33). The strength and the kinetics of the swelling reaction correlate with the induction and recruitment of T cells to the site of infection. Naïve mice show a strong swelling reaction on day 8 after infection, whereas LCMV-immune mice immediately clear the virus and therefore fail to generate a strong swelling reaction. To assess priming and homing of T cells in op<sup>−/−</sup> mice, 100 PFU LCMV WE were injected into the footpad of op<sup>−/−</sup> and C57BL/6 control mice (Fig. 1). While C57BL/6 mice generated a strong swelling reaction on day 8, op<sup>−/−</sup> mice failed to produce a swelling reaction, suggesting that in op<sup>−/−</sup> mice LCMV either did not induce a T cell response or induced T cells did not migrate to the footpad. To evaluate whether op<sup>−/−</sup> mice generated LCMV-specific CTL responses measurable in vitro, cytotoxicity assays were performed with spleen cells from op<sup>−/−</sup> and C57BL/6 mice infected i.f. with LCMV (Fig. 2A). The data show that op<sup>−/−</sup> mice generated slightly reduced but still strong CTL responses. Thus, the lack of a footpad swelling reaction in op<sup>−/−</sup> mice cannot be explained by the lack of CTL induction but may rather reflect the failure of CTL homing to the footpad.

op<sup>−/−</sup> mice are unable to control LCMV during the acute phase of the infection

A possible explanation for the homing deficiency of CTL into the footpad is that virus spreads throughout all organs and recruits CTL away from the site of infection. To assess this possibility, virus titers in op<sup>−/−</sup> mice were determined in different organs of mice infected i.f. with 100 PFU LCMV (Fig. 2B). LCMV titers were 10- to 50-fold increased in thymus, spleen, and liver of op<sup>−/−</sup> mice compared with C57BL/6 controls, suggesting that op<sup>−/−</sup> mice could not efficiently control virus spread during the acute phase. This observation was even more pronounced when 100 PFU LCMV were injected i.v., in which case virus titers in op<sup>−/−</sup> mice were 100–10,000 times higher than in C57BL/6 controls: thymus op, 2.68 × 10<sup>6</sup> ± 9.1 × 10<sup>5</sup> PFU; thymus B6, 1.1 × 10<sup>4</sup> ± 8.9 × 10<sup>3</sup> PFU; liver op, 4.9 × 10<sup>5</sup> ± 2.6 × 10<sup>4</sup> PFU; liver B6, 3.1 × 10<sup>3</sup> ± 2.9 × 10<sup>2</sup> PFU; spleen op, 2.5 × 10<sup>6</sup> ± 2.2 × 10<sup>5</sup> PFU; spleen B6, 1.2 × 10<sup>4</sup> ± 1 × 10<sup>4</sup> PFU. Taken together, uncontrolled virus dissemination may lead to increased Ag loads in peripheral organs and deviation of CTL into these organs.

FIGURE 3. A, A proportion of op<sup>−/−</sup> mice recovering from generalized immunopathology become virus carriers. Three groups of a total of 11 op<sup>−/−</sup> mice were injected i.f. with 100 PFU LCMV. Each group was tested in a separate experiment. The LCMV titers in the indicated organs were determined from eight surviving mice (op) from the groups described in A and the C57BL/6 control (B6) mice. Virus titers were measured in the brain. Expt. 1, Virus spreads to the brain in op<sup>−/−</sup> mice. Three op<sup>−/−</sup> and C57BL/6 mice were injected i.v. with 2 × 10<sup>6</sup> PFU VSV. On the indicated days neutralizing IgM plus IgG and IgG titers were determined. One of three independent experiments is shown. B, exp. 1, Virus spreads to the brain in op<sup>−/−</sup> mice. Three op<sup>−/−</sup> mice failed to generate a footpad swelling reaction due to a biphasic CD8<sup>+</sup> and CD4<sup>+</sup> T cell response (29, 33). The strength and the kinetics of the swelling reaction correlate with the induction and recruitment of T cells to the site of infection. Naïve mice show a strong swelling reaction on day 8 after infection, whereas LCMV-immune mice immediately clear the virus and therefore fail to generate a strong swelling reaction. To assess priming and homing of T cells in op<sup>−/−</sup> mice, 100 PFU LCMV WE were injected into the footpad of op<sup>−/−</sup> and C57BL/6 control mice (Fig. 1). While C57BL/6 mice generated a strong swelling reaction on day 8, op<sup>−/−</sup> mice failed to produce a swelling reaction, suggesting that in op<sup>−/−</sup> mice LCMV either did not induce a T cell response or induced T cells did not migrate to the footpad. To evaluate whether op<sup>−/−</sup> mice generated LCMV-specific CTL responses measurable in vitro, cytotoxicity assays were performed with spleen cells from op<sup>−/−</sup> and C57BL/6 mice infected i.f. with LCMV (Fig. 2A). The data show that op<sup>−/−</sup> mice generated slightly reduced but still strong CTL responses. Thus, the lack of a footpad swelling reaction in op<sup>−/−</sup> mice cannot be explained by the lack of CTL induction but may rather reflect the failure of CTL homing to the footpad.

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After LCMV infection, \( \text{op}^{-/-} \) mice developed a generalized immunopathology

Mice that cannot efficiently control virus spread show signs of generalized immunopathological disease. To evaluate whether \( \text{op}^{-/-} \) mice eventually controlled LCMV infection, they were infected i.f., with LCMV and clinical symptoms of generalized immunopathology were monitored. In contrast to C57BL/6 mice, all LCMV-infected \( \text{op}^{-/-} \) mice developed generalized immunopathology characterized by a hunched back, ruffled fur, and lethargy: 9 of 25 \( \text{op}^{-/-} \) mice succumbed to the infection between days 12 and 18, and the remaining mice recovered completely from clinical symptoms by days 21–28. Thus, \( \text{op}^{-/-} \) mice are immunocompromised and have difficulties controlling virus spread despite being able to generate significant primary CTL responses.

**Impaired virus clearance in \( \text{op}^{-/-} \) mice correlates with the lack of LCMV-specific CTL**

To determine whether \( \text{op}^{-/-} \) mice, which recover from transient immunopathology, eliminate the virus or become virus carriers due to CTL exhaustion (34–36), a total of 11 \( \text{op}^{-/-} \) mice were injected with LCMV into the footpad, virus titer were determined in the organs between days 21 and 28, and the presence of CTL was assessed in secondary in vitro assays. Three of the \( \text{op}^{-/-} \) mice died with severe clinical symptoms of generalized immunopathology. Of the remaining eight \( \text{op}^{-/-} \) mice, six mice failed to eliminate LCMV from all organs (Fig. 3A) and two mice completely cleared the virus below detectable levels (data not shown). After in vitro restimulation, LCMV-specific CTL could be recovered from the mice that had eliminated the viruses (Fig. 3B), whereas no LCMV-specific CTL could be recovered from the carrier mice (Fig. 3C). Thus, elimination of virus correlated with CTL activity, whereas impaired virus clearance correlated with the absence of LCMV-specific CTL.

**FIGURE 5.** A–F. Macrophage and FDC populations in spleen and lymph nodes of \( \text{op}^{-/-} \) mice. Spleen (A, C, D, and F) and lymph node (B and E) sections from C57BL/6 (A–C) and \( \text{op}^{-/-} \) (D–F) mice were stained with an Ab recognizing mouse metallophilic macrophages (MOMA) or FDCs (4C11). G–J, Distribution of viral Ag in the spleen of \( \text{op}^{-/-} \) mice. C57BL/6 (G and H) and \( \text{op}^{-/-} \) (I and J) mice were injected i.v. with 5 \( \times \) 10^6 PFU VSV. After 6 h spleens were removed and sections were stained for VSV Ag (G and J) and FDC (4C11; H and J). Data shown are representative of one mouse from three independent mice.

VSV Ag is efficiently recruited to the spleen

Recruitment of VSV by natural Abs to secondary lymphoid organs plays a key role in controlling disease by reducing virus spread and enhancing induction of an immune response (22, 38). \( \text{op}^{-/-} \) mice lack metallophilic MZM in the spleen and subcapsular macrophages in the lymph nodes, and have normal FDC populations (Fig. 5, upper panels). To investigate whether VSV was efficiently recruited to the spleen, \( \text{op}^{-/-} \) mice were infected i.v. with 5 \( \times \) 10^6 PFU VSV. No difference in VSV titers was found in the spleens after 6 h of infection. Titer were >2.1 \( \times \) 10^6 PFU per gram of organ in both \( \text{op}^{-/-} \) and control mice. Consistent with this, cryosections revealed that VSV Ag was recruited to the spleen. However, while VSV Ag was predominantly found in the marginal zone of C57BL/6 mice, VSV Ag could be found in the red pulp.
and the white pulp of op−/− mice. Moreover, the Ag largely colocalized with FDC in op−/− mice (Fig. 5).

Discussion

For non- or low-cytopathic viruses the relative balance between virus spread and CTL response critically defines whether the virus will be cleared, immunopathology will ensue, or the immune response will be exhausted and the virus will persist (39). Because cytopathic viruses directly destroy infected cells, they cannot establish a carrier state without killing the host; therefore, virus spread has to be controlled rapidly.

Early CTL and B cell responses against LCMV and VSV were largely unimpaired in op mice. The small reduction in the early day 8 cytotoxic activity of spleen cells against LCMV in op−/− mice is probably a consequence of virus dissemination and CTL extravasation into peripheral infected tissues rather than inefficient CTL priming. This view is consistent with the observation that op−/− mice failed to generate a footpad swelling reaction, probably due to CTL recruitment to many sites in addition to the infected footpad (40).

All op−/− mice went through a critical phase of generalized immunopathology, which one-third of the mice did not survive. In a small proportion of the op−/− mice an efficient CTL response dominated over virus spread, virus was cleared, and LCMV-specific CTL could be recovered. However, most of the mice failed to completely clear LCMV from all organs (virus persisted particularly in the kidney) and no LCMV-specific CTL could be recovered, indicating that CTL had been exhausted. It has previously been observed that T cell-dependent LCMV clearance involves cooperation between T cells and probably macrophages (14). In addition, an earlier study by Seiler et al. (18) investigated the role of MZM in immunity against LCMV. The authors depleted MZM by administration of liposomencapsulated clodronate (15–17) and reported that CTL responses were exhausted in all mice. There was no mention of generalized immunopathology, most likely because the treatment of mice with clodronate liposomes is stressful to the animal and clinical symptoms may have been masked due to this harsh treatment. In this work we show that three clinical outcomes are possible: 1) virus elimination accompanied by intact LCMV-specific CTL responses, 2) virus persistence and CTL exhaustion, or 3) death as a consequence of virus spread and immunopathological CTL response.

After infection with VSV, op−/− mice generated normal neutralizing (IgM plus IgG) and IgG responses; nevertheless, mice eventually succumbed to disease. Analysis of VSV titers in the brain revealed that virus spread could not be controlled in these mice and that VSV had disseminated to the brain. This was apparent with an infectious dose of 2 × 106 PFU of VSV; however, a 100-fold lower dose was controlled. Natural Abs and complement have been implicated in directing Ag to lymphoid organs to accelerate and enhance immune responses (22, 38, 41); however, the precise mechanism by which virus spread is inhibited and influenced by MZM is unknown. VSV is sensitive to type I IFNs and thus early IFN responses generated by MZM may account for this inhibition. In op−/− mice, VSV Ag was recruited into the white pulp of the spleen and colocalized with FDC, while in C57BL/6 mice Ag localized to the marginal zone. Thus, Ag recruitment was still possible in op−/− mice, but to a different site. Additional experiments will be required to identify by which receptors VSV Ag is captured on FDC. Nevertheless, it seems clear that the Ag absorbance and removal capacity of the FDC are not sufficient to prevent viral dissemination.

op−/− mice have been shown to generate normal MLR T cell responses and normal B cell responses in vivo against OVA (12). The present study is consistent with these findings. However, despite generating normal T and B cell responses, op−/− mice were susceptible to viral infection. This points to the importance of the architecture of lymphoid organs for controlling viral infections in vivo (42–45). Crucial functions of structural components of lymphoid organs may not be uncovered if nonreplicating Ags are used. Although such experimental systems provide us with important information, they usually disclose rather a narrow window of the complex processes that are triggered by infections.

Taken together this study shows that the early innate immune system provides an important preparatory phase for the adaptive immune system: cooperation between the innate and the adaptive immune system shifts the balance between induction of an immune response and virus spread in favor of the immune response. This is particularly important for cytopathic viruses where virus spread usually leads to death, while non- or low-cytopathic viruses, depending on the relative balance between immune response and virus spread, can establish a virus carrier state without harming the host if extensive immunopathology is avoided.

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