Role of spleen macrophages in innate and acquired immune responses against mouse hepatitis virus strain A59

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SUMMARY

Owing to their scavenging and phagocytic functions, spleen macrophages are regarded to be important in the induction and maintenance of both innate and acquired immune defence mechanisms. In this study, we investigated the role of spleen macrophages in immunity against mouse hepatitis virus strain A59 (MHV-A59). Previous studies showed that spleen and liver macrophages are the first target cells for infection by MHV-A59 in vivo, suggesting that they could be involved in the induction of immune responses against MHV-A59. We used a macrophage-depletion technique to deplete macrophages in vivo and studied the induction of virus-specific antibody and cytotoxic T-cell (CTL) responses and non-immune resistance against MHV-A59 in normal and macrophage-depleted mice. Virus titres in spleen and liver increased rapidly in macrophage-depleted mice, resulting in death of mice within 4 days after infection. Elimination of macrophages before immunization with MHV-A59 resulted in increased virus-specific humoral and T-cell proliferative responses. However, virus-specific CTL responses were not altered in macrophage-depleted mice. Our results show that spleen macrophages are of major importance as scavenger cells during MHV-A59 infection and are involved in clearance of virus from the host. In addition, macrophages may be involved in the regulation of acquired immune responses. In the absence of macrophages, increased virus-specific T-cell and antibody responses are detectable, suggesting that macrophages suppress MHV-A59-specific T- and B-cell responses and that other cells serve as antigen-presenting cells.

INTRODUCTION

Innate and acquired immune mechanisms collaborate to allow the host to resist invading pathogens. Macrophages are thought to be involved in both immune defence mechanisms.1 By ingestion and digestion of micro-organisms, they may be able to remove a substantial percentage of foreign invaders such as bacteria, viruses and yeast cells from the circulation and organs of the host.2,3 In addition to these scavenging and phagocytic functions, macrophages are also regarded as important antigen-presenting cells (APC) for the induction of specific acquired immune responses.4 Evidence has been presented that spleen macrophages play a crucial role in the induction of humoral immunity against particulate antigens such as sheep red blood cells (SRBC).5,6 Recent studies have revealed that macrophages also play a role as APC in the induction of cytotoxic T lymphocyte (CTL) responses. Debréck et al.7 showed that the induction of CTL responses to antigens associated with cell debris is dependent on the presence of macrophages as accessory cells. Furthermore, Zhu et al.8 showed that CTL responses to liposomal antigens were completely dependent upon the presence of spleen macrophages. In addition, it has been shown that in some viral infections, presence of macrophages is necessary to develop virus-specific CTL.9

Our studies concern the role of spleen macrophages in the different mechanisms of host resistance against viral infections. As a model, we used mouse hepatitis virus strain A59 (MHV-A59). MHV-A59 belongs to the family Coronaviridae and causes a variety of acute and chronic infections in mice and rats, ranging from acute hepatitis and encephalomyelitis to chronic demyelination.10,11 In previous studies, Heemskerk et al.12 demonstrated that infection of mice with MHV-A59...
leads to the generation of CD4\textsuperscript{+} major histocompatibility complex (MHC) class II-restricted virus-specific CTL. Histological studies showed that macrophages are the first cells infected with MHV-A59 in vivo,\textsuperscript{15} suggesting that macrophages could be involved in the induction of specific immune responses against MHV-A59.

The aim of the present study was to investigate the role of spleen macrophages in the induction of virus-specific CTL responses, in the induction of MHV-A59-specific antibody responses, and in non-immune resistance against MHV-A59. In order to study the role of macrophages in any of these defence mechanisms against MHV-A59, we used the recently developed liposome-mediated macrophage ‘suicide’ technique.\textsuperscript{16} This technique is based on the liposome-mediated cytosolic delivery and accumulation of drugs that disturb the metabolism of macrophages, ultimately leading to irreversible damage and depletion of macrophages from tissues. We were able to show that spleen macrophages are involved in clearance of the virus, but suppress MHV-A59-specific T- and B-lymphocyte responses.

**MATERIALS AND METHODS**

**Mice**
Specific pathogen-free (including seronegative for MHV) male BALB/c mice were obtained from the central animal house (GDK, Utrecht, the Netherlands) and used at 6-8 weeks of age. Mice were kept in filter top cages with free access to commercial mouse food and tap water.

**Virus**
MHV-A59, a virulent hepatotropic strain, and the less virulent temperature-sensitive mutant of MHV-A59, ts342, were propagated on Sac(-) cells and virus stocks were prepared as described previously.\textsuperscript{17,18} Inactivated virus was prepared by ultraviolet (UV) irradiation for 10 min.

**Cell lines**
The A20 cell line, an H-2\textsuperscript{b} expressing B-cell lymphoma [American Type Culture Collection (ATCC), Rockville, USA], was used as the target cell in the cytoxicity assays.

**Preparation of dichloromethylene diphosphonate-loaded liposomes**
Multilamellar liposomes were prepared as previously described.\textsuperscript{19} Briefly, 86 mg phosphatidylcholine (Lipoid GmbH, Ludwigsafen, Germany) and 8 mg cholesterol (Sigma, St Louis, MO) molar ratio 6:1, were dissolved in chloroform in a round bottom flask. The thin film formed on the interior of the flask after low-vacuum rotary evaporation at 37\textdegree\ C was dispersed in 10 ml phosphate-buffered saline (PBS) containing 1.99 g dichloromethylene diphosphonate (Cl\textsubscript{2}MDP), a kind gift of Boehringer Mannheim GmbH, Mannheim, Germany by gentle rotation for 10 min. Free Cl\textsubscript{2}MDP was removed by rinsing the liposomes with sterile PBS and centrifuging them for 30 min at 25 000 \texttimes\ g at 16\textdegree\ C. Finally, the liposomes were resuspended in 4 ml PBS. For depletion of spleen macrophages [red pulp macrophages (RPM), marginal zone macrophages (MZM) and marginal metaphilic macrophages (MMM)] and liver macrophages, 0.1 ml of the suspension was injected intravenously (i.v.) per 10 g body weight.

**Immunization protocol**
Previous studies have shown that i.v. infection of BALB/c mice with MHV-A59 results in acute hepatitis and death of all mice within 7 days.\textsuperscript{14} Therefore, mice were preimmunized with 10\textsuperscript{5.9} plaque-forming units (PFU) of a less virulent temperature-sensitive mutant (ts342) of MHV-A59 and 7 days later boosted with 5 \times 10\textsuperscript{5} PFU wild-type MHV-A59 (wtMHV-A59). When indicated, spleen and liver macrophages were depleted by one i.v. injection of Cl\textsubscript{2}MDP-loaded liposomes 2 days before administration of ts342 and 2 days before boosting with wtMHV-A59. In other experiments, mice were infected with 4 \times 10\textsuperscript{5} PFU wtMHV-A59, and when indicated, spleen and liver macrophages were depleted 2 days before infection with a single i.v. injection of Cl\textsubscript{2}MDP-liposomes.

**Lymphocyte proliferation assay**
Lymphocyte proliferation assays were performed 3 weeks after immunization in 96-well flat-bottom plates in triplicate cultures. Each well contained 2 \times 10\textsuperscript{5} spleen cells and various concentrations of UV-irradiated MHV-A59 in 0.2 ml of ICS medium in 96-well U-shaped plates and incubated for 72 hr. Irradiation of infected cells had no influence on the lytic potential of immune cells. Virus stocks were prepared as described previously.\textsuperscript{19} Inactivated virus was prepared by UV irradiation for 10 min. Cytotoxicity assay

**Generation of MHV-A59-specific CTL in bulk culture**
Three weeks after immunization, spleen cells of immunized mice were isolated and stimulated in bulk culture (2 \times 10\textsuperscript{7}) with ICS medium and 1.25 \times 10\textsuperscript{5} irradiated (3,000 rads) MHV-A59-infected syngeneic spleen cells multiplicity of infection (MOI) of 0.3) for 5 days in 15 ml complete IMDM.

**Cytotoxicity assay**
Varying numbers of spleen cells stimulated in vitro were added to 5 \times 10\textsuperscript{4} SeCrO\textsubscript{4} labelled target cells in 0.15 ml complete IMDM in 96-well U-shaped plates and incubated for 5 hr at 37\textdegree\ C and 5% CO\textsubscript{2}. Prior to labelling with Na\textsubscript{18}CrO\textsubscript{4}, target cells were infected for 3 hr at 37\textdegree\ C with MHV-A59 (MOI of 50). After 5 hr incubation, 100 \muCi superantigen was assayed for 18\textsubscript{Cr}. Results are presented as percentage specific lysis defined as [(experimental lysis)-(spontaneous lysis)]/(total lysis)-(spontaneous lysis)]. Maximum spontaneous release values were always <20% of total lysis.

**Enzyme-linked immunosorbent assay (ELISA)**
Anti-MHV-A59 antibodies in serum of infected mice were detected by a direct ELISA. Microtitre 96-well plates (Nunc, Denmark) were coated for 3 hr at 37\textdegree\ C and overnight at 4\textdegree\ C with UV-inactivated MHV-A59, 10\textsuperscript{5} PFU/well, diluted with coating buffer (0.1 M NaHCO\textsubscript{3}, pH 9.6). Plates were washed three times with washing buffer (0.05% Tween-20 in PBS) followed by blocking with 10% Prolifer milkpowder solution (Nutricia, Zoetermeer, the Netherlands) for 1 hr at 37\textdegree\ C. Next, the plates were washed three times and incubated with serial dilutions of test sera (50 \mu l/well) for 2 hr at 37\textdegree\ C. As a control, normal

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mouse serum (NMS) was used. All samples were tested in duplicate. After washing three times, either biotinylated rabbit anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b or IgG3 (Zymed, CA) was added (50 μl/well) and the plates were incubated for 2 hr at 37°. Subsequently, plates were washed and incubated with peroxidase-conjugated streptavidin (DAKO, Denmark) for 2 hr at 37°. As a substrate, ortho-phenylene-diamine-dihydrochloride (OPD, Sigma) (50 μl/well) with freshly added H₂O₂ was used. The reaction was stopped after 10 min by addition of 1 ml H₂SO₄. The absorbance was measured by ELISA reader (Organon Technika, the Netherlands) at 492 nm.

**RESULTS**

**Macrophages play a major role in clearance of MHV-A59**

To determine the role of macrophages in innate immunity to MHV-A59, normal and macrophage-depleted mice were infected with 4 x 10⁶ PFU wtMHV-A59, and the virus titres in liver and spleen were determined at the indicated time-points. Figure 1 shows the survival of mice after infection. Normal mice died of MHV-A59 infection 5–7 days after infection of macrophage-depleted mice 12 hr after infection, and raised to approximately 10⁷ TCID₅₀ in macrophage-depleted mice and to 5 x 10⁶ TCID₅₀ in normal mice within 4 days after infection. At the time-point at which the normal mice died, the virus load in the spleen had reached the same level as in the macrophage-depleted mice on day 4. These results show that macrophages in the spleen and the liver serve an important scavenging function in the defence against MHV-A59, and could suggest that they play a role in the induction of specific immune responses.

**Effect of macrophage depletion of viral titres in liver and spleen.** Normal (hatched blocks) and macrophage-depleted (shaded blocks) mice were infected with 4000 PFU wtMHV-A59 and at the indicated time-points viral titres in livers and spleens of five mice were determined by an end-point dilution assay. Data are expressed as mean TCID₅₀ per 200 mg liver or 50 mg spleen. SD were always <7% of the mean TCID₅₀. Shown is one representative out of three performed experiments.

**Figure 1.** Removal of macrophages before infection with wtMHV-A59 results in early death. Groups of 10 normal (●) or macrophage-depleted (▲) mice were infected i.v. with 4000 PFU wtMHV-A59. Results are one representative out of three performed experiments.
Spleen macrophages and response to mouse hepatitis virus

Figure 3. Effect of macrophage depletion on MHV-A59-induced proliferation. Spleen cells from normal (hatched blocks, n = 5) and macrophage-depleted mice (shaded blocks, n = 5) were tested for MHV-A59-induced proliferative responses 3 weeks after immunization with MHV-A59. Each well contained 2 x 10⁵ splenocytes and indicated concentrations (UV-irradiated) MHV-A59. Shown are SIs as mean ± SD. In the absence of virus, 2335 ± 195 c.p.m. were measured. *P < 0.001, Student's t-test.

ability to proliferate upon antigen-specific stimulation, resulting in significantly higher SI. When macrophages were eliminated 2 days before immunization with ts342 only, or 2 days before boosting with wtMHV-A59 only, an approximately twofold increase in proliferative responses was observed in spleen cell cultures as compared with spleen cell cultures from normal mice (results not shown). In addition, when T-cell proliferation was measured in spleen cell cultures from normal and macrophage-depleted mice immunized with ts342 only, a twofold increased SI was measured in cultures from macrophage-depleted mice compared with normal mice when UV-irradiated ts342 was used as antigen (results not shown). These results show that in the absence of spleen macrophages antigen-specific T-cell proliferation is increased, possibly as a result of increased virus load.

In contrast to the MHV-A59-induced proliferation, macrophage depletion before immunization had no effect on the cytotoxic capacity of the MHV-A59-specific T cells. Figure 4. shows the cytolytic response of effector cells stimulated in vitro to MHV-A59-infected A20 cells, which were used as target cells. The CTL bulk cultures of both normal and macrophage-depleted mice showed an equal cytotoxicity towards MHV-A59-infected target cells, even though we were able to recover more viable cells from the cultures of macrophage-depleted mice (results not shown). These results suggest that spleen macrophages are involved in the regulation of proliferative T-cell responses to MHV-A59 infection, but not in CTL induction.

Absence of macrophages results in increased antibody titres in MHV-A59-immunized mice

To further study the effect of macrophage depletion on the induction of a MHV-A59-specific immune response, we ana-

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Figure 4. Macrophage depletion does not affect induction of MHV-A59-specific CTL response. Spleen cells from normal (○, n = 5) and macrophage-depleted mice (♀, n = 5) were cultured for 5 days with MHV-A59-infected APC. Viable cells were harvested and pooled and tested for their capacity to kill MHV-A59-infected A20 target cells in a 51Cr-release assay. Open symbols (○, ♀) represent killing of non-infected target cells, closed symbols (●, □) represent killing of MHV-A59-infected target cells. Shown is one representative out of five performed experiments.

Figure 5. Effect of macrophage depletion on induction of humoral immune response to MHV-A59. Immunization of mice with ts342 and MHV-A59 induced mainly IgG2a anti-MHV-A59 antibodies (Fig. 5). Depletion of macrophages before immunization resulted in significantly increased antibody titres and no shift in isotype was observed. MHV-A59-specific antibody titres increased in time, and the same difference in specific antibody titre between normal and macrophage-depleted mice remained for at least 4 weeks after immunization.
in vitro the 5-day normal mice. However, their capacity to lyse virus-infected depleted mice may be the result of the absence of malfunctioning APC. Removal of macrophages from the spleen and liver before immunization with MHV-A59 results in increased virus-specific humoral and cellular immune responses. In the experiments described here we used the liposomemediated macrophage depletion technique to deplete macrophages from tissues in vivo. It has previously been shown that after a single i.v. injection of the Cl₃MDP-liposomes removal of macrophages from the liver is complete. In the spleen however, only the RPM, MMM and MZM are affected by the liposomes. Within the white pulp area, the white pulp macrophages (WPM) and also the dendritic cells (DC) are not depleted. In earlier studies we have shown that macrophages in the spleen and liver are the first cells infected with MHV-A59.²³ However, MHV-A59 could also be detected in DC, WPM and B lymphocytes. Since it is known that not only macrophages but also DC function as APC for viral antigens in vivo,²⁵ and since we cannot deplete these cells from the spleen with the macrophase depletion technique, we cannot exclude a possible role for DCs and WPM in the induction of MHV-A59-specific immune responses. Furthermore, repopulation of RPM in the spleen and Kupffer cells in the liver is complete within 14 days after depletion.²⁶ Since accessory cell function of these macrophages will be inferior to DC, circulating virus titres may already have been reduced to very low levels and priming of MHV-specific T cells will have occurred by then, a role for RPM in the induction of MHV-A59-specific immune responses is unlikely, but cannot be disregarded. Repopulation of splenic MMM and MZM is only complete 6 weeks and > 2 months after depletion, respectively, and these macrophages are therefore definitively not involved in the induction of MHV-A59-specific immune responses. The results of the proliferation assays showed that spleen cells from macrophage-depleted mice had an enhanced proliferative response to MHV-A59 compared with spleen cells from normal mice. However, their capacity to lyse virus-infected cells was not altered. This may be due to the fact that after the 5-day in vitro culture, all cells possess maximum cytotoxic capacity, which is detected in the ⁵¹Cr-release assay. Whether depletion of macrophages before infection results in increased frequency of MHV-A59-specific CD4⁺ T cells or increased activation of the T cells only, remains to be investigated. The fact that we were able to recover more viable cells from the bulk cultures of macrophage-depleted mice might be a reflection of their increased virus-specific proliferative responses. The increased activation of CD4⁺ T cells was also reflected in the induction of MHV-A59-specific antibodies. Serum titres were significantly increased in the macrophage-depleted mice, and remained elevated at least 4 weeks after immunization (results not shown), which may be the result of improved B-cell help from the CD4⁺ T cells. Previously, van Rooijen²⁷ reported that macrophages are not required for the induction of antibody responses against small antigens, such as soluble proteins and thymus-independent antigens. These studies showed that removal of macrophages before immunization with such antigens resulted in increased antigen-specific antibody titres, which may be explained by competition for the antigen by macrophages and other APC. Here, we report increased specific immune responses to viral antigens after macrophage elimination, which may be the result of increased viral antigen load in macrophage-depleted mice. We showed that macrophage depletion results in increased virus titres in spleen and liver after infection with wtMHV-A59. In addition, we have shown that in macrophage-depleted mice immunized with ts342 only, enhanced proliferative T-cell responses to ts342 are observed. This may be the result of the fact that in macrophage-depleted mice, less virus is cleared from the circulation; therefore more viral antigen is available for other APC, such as DC, to present to T cells, resulting in increased immune responses. However, it has to be considered that although viruses may be regarded as small particulate antigens for which macrophages and other APC may compete, the immune response in macrophage-depleted mice may be altered as a result of cytokine production by virus-infected cells. Several research groups have reported altered immune responses in MHV-infected mice. Coutelier et al.²⁸ showed that MHV-A59 infection before immunization with a protein antigen alters the isotype distribution of the anti-protein antibodies. Other groups reported depletion of T- and B-cell subpopulations from lymphoid organs, impaired T-cell responses to mitogens, changed patterns in cytokine production by T cells, altered mucosal immune responses and suppressed host resistance to secondary viral infections.²⁹ ³⁰ However, the suppressive mechanism is not clear. One explanation for the immunomodulation could be the presence of malfunctions. Activation of T cells requires specific T-cell receptor signalling in combination with secondary signals from APC.³¹ In the absence of appropriate co-stimulating signals, T cells become unresponsive. Infection of macrophages with MHV affects their function and phenotype, which is reflected by enhanced phagocytic and cytolytic activity and altered cytokine levels.³² ³³ More importantly, it has been shown that APC function of macrophages from the spleen and peritoneal cavity of MHV-infected mice is impaired.³⁴ The increased immune response we observed in macrophage-depleted mice may be the result of the absence of malfunctioning APC, i.e. MHV-infected macrophages, in these mice. We and others have shown that other potential APC, such as B cells and DC in the spleen, express receptors for MHV-A59 and are infected by MHV as well and these cells are not depleted by the Cl₃MDP-liposomes.³⁵ ³⁶ It has been shown that the APC function of B cells is hardly affected by MHV infection, whereas not much is known about the effect on DC.³⁷ In addition, DC have been shown to function much better than macrophages as accessory cells for T-cell stimulation.³⁸ Therefore, the possibility exists that in the absence of macrophages, DC and B cells function as APC resulting in an increased immune response. Further experiments will be designed to prove whether our hypothesis is correct. Alternatively, the suppressed immune response in MHV-A59-infected mice could be the result of the production of
immunosuppressive mediators by MHV-infected macrophages such as prostaglandins. It has been shown that prostaglandin E (PGE) suppresses humoral and cellular immune responses. Lahm & Virelizier suggested that prostaglandins mediate the suppression of antibody production in MHV-infected mice after they showed that indomethacin treatment, which inhibits PGE synthesis, enhanced antibody responses in MHV-infected mice. In addition, cytokines, such as interferon-α and -β, produced by macrophages upon infection with MHV, may suppress T-cell responses.23 On the other hand, virus infection may modulate normal cytokine production by macrophages resulting in altered immune responses. Recently it was shown that the suppression of immune responses during measles virus infection is due to down-regulation of interleukin-12 (IL-12) production, a critical cytokine for the development of cell-mediated immune responses.34 Whether production of IL-12 by MHV-infected macrophages remains to be investigated. However, we suggest that in the macrophage-depleted mice, production of mediators such as PGE is absent which may result in increased immune responses.

Although MHV-infected macrophages suppressed the induction of a specific immune response, they are of major importance in protection of the host against wMHV-A59. Removal of macrophages from tissues before infection with wMHV-A59 resulted in exacerbation of the viral infection with elevated virus titres in spleen and liver and increased mortality rates. We did not determine how elimination of macrophages from spleen and liver affected virus titres in other compartments, such as blood and lymph nodes. Virus uptake in these organs may have been enhanced in macrophage-depleted mice, as a result of increased circulating virus titres. However, uptake of MHV-A59 by macrophages in those compartments is not sufficient to clear the virus and prevent the onset of hepatitis. This important role for macrophages as primary scavenger cells for viruses to control infections has been shown in other virus infections in mice, such as West Nile virus, extromelia virus and yellow fever virus.25 Macrophages may play a significant role in restriction of virus replication during infection for several reasons. After ingestion of a virus particle, macrophages inactivate the virus by digestion. Furthermore, activated macrophages secrete cytokines and mediators such as interferon-α and -β, IL-12 and tumour necrosis factor-α, which may make surrounding cells insensitive for virus infection and may activate NK cells and T lymphocytes.26 Thirdly, macrophages may act as non-specific cytotoxic cells and lys virus-infected cells.

In conclusion, we have shown that MHV-A59-infected macrophages from the spleen suppress the induction of a virus-specific immune response, probably due to a combination of removal of viral antigen from the circulation, impaired accessory cell activity and the secretion of immunosuppressive mediators. However, spleen and liver macrophages are of major importance as scavenger cells in the non-immune defence against MHV-A59 and may protect mice from developing hepatitis by restricting virus replication.

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