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Role of virus-specific CD4$^+$ cytotoxic T cells in recovery from Mouse Hepatitis Virus infection

O. L. C. WIJBURG,* M. H. M. HEEMSKERK,†‡ A. SANDERS,* C. J. P. BOOG†§ & N. VAN ROOIJEN*
**Department of Cell Biology and Immunology, Faculty of Medicine, Vrije Universiteit, Amsterdam and †Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, the Netherlands*

SUMMARY

Macrophages and T lymphocytes play an important role in recovery from viral infections. During mouse hepatitis virus (MHV-A59) infection, a clear virus-specific class II-restricted cytotoxic T-cell response is generated. Transfer of these CD4$^+$ cytotoxic T cells (CTL) into naive mice protects against a lethal challenge with MHV. However, their *in vivo* antiviral effector mechanism is not yet clear. To further investigate a possible effector mechanism, we studied the effect of adoptive transfer of CD4$^+$ CTL on virus localization in spleen and liver. We showed that adoptive transfer of virus-specific T cells does not affect localization of MHV-A59 in different macrophage subsets. Interestingly, a rapid and large infiltrate of CD4$^+$ T cells in and around MHV-A59-infected foci in the liver was observed early in infection, whereas no CD8$^+$ T cells were detectable. Moreover, transfer of virus-specific T cells resulted in significantly decreased viral titres in the liver and spleen and a marginally increased anti-MHV-A59 IgM production. These results imply an important role for virus-specific CD4$^+$ CTL in elimination of infectious MHV-A59 and induction of an effective immune response in the absence of CD8$^+$ CTL.

INTRODUCTION

During viral infections, both non-specific and specific effector mechanisms collaborate in order to induce an effective immune response, leading to clearance of virus from the host and induction of long-lasting virus-specific memory. Non-specific defense mechanisms involve phagocytosis of viral particles by macrophages resulting in clearance of viruses from infected tissues. On the other hand, T lymphocytes have been shown to play a crucial role in the cell-mediated specific immune response against virus infections.[1] In various mouse models, it has been shown that either CD4$^+$ or CD8$^+$ cytotoxic T lymphocytes (CTL) are able to lyse virally infected cells *in vitro* and promote recovery from viral infections *in vivo*.[2–4] Cytokines produced by virus-specific T cells, such as interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), have been suggested to play a role in viral clearance[5] as well.

Our studies concern the role of macrophages and T cells in the induction and maintenance of the specific cell-mediated immune response against mouse hepatitis virus strain A59 (MHV-A59) infection. MHV-A59 causes a variety of acute and chronic infections in mice and rats, ranging from acute hepatitis and encephalomyelitis to chronic demyelination.[6,7] Recently, Heemskerk *et al.*[8] have characterized the CTL response during MHV-A59 infection. They showed that MHV-A59 infection of BALB/c and C57/BL6 mice leads to the activation of virus-specific major histocompatibility complex (MHC) class II-restricted CD4$^+$ CTL. Clones of these CD4$^+$ T cells were able to lyse virally infected targets *in vitro* and showed a T-helper-1 (Th-1) phenotype.[8,9] Moreover, adoptive transfer studies using the MHV-A59 specific CTL clone HS1 showed a significant protection of mice (60–80%) against a lethal challenge with MHV-A59.

The aim of the present study was to determine localization of MHV-A59 in spleen and liver of mice and, in addition, to study the *in vivo* antiviral effector mechanisms of the CD4$^+$ CTL. We studied the effect of adoptive transfer of the HS1 CTL clone on localization of MHV-A59 in spleen and liver, elimination of infectious virus from the liver and spleen, and induction of anti-MHV-A59 antibodies.

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Abbreviations: APC, antigen presenting cell; CTL, cytotoxic T lymphocytes; DC, dendritic cell; MMM, marginal metallophilic macrophage; MZM, marginal zone macrophage; mAb, monoclonal antibody; MHV, mouse hepatitis virus; NMS, normal mouse serum; RPM, red pulp macrophage; WPM, white pulp macrophage.

‡Present address: Department of Immunology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands.

§Present address: Department of Transplantation Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P.O. Box 9190, 1006 AD Amsterdam, the Netherlands.

Correspondence: Dr O. L. C. Wijburg, Dept of Cell Biology and Immunology, Faculty of Medicine, Vrije Universiteit, Amsterdam, the Netherlands.

MATERIALS AND METHODS

Mice

Specific pathogen-free (including seronegative for MHV) male BALB/c mice were obtained from the central animal house (GDL, Utrecht, the Netherlands) and used at 6–10 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, was propagated on Sac(-) cells and virus stocks were prepared as has previously been described.[10] Inactivated virus was prepared by UV irradiation for 10 min.

T-lymphocyte cultures

The isolation, maintenance and properties of the MHV-A59-specific CTL clone HS1 has previously been described.[8] Briefly, HS1 cells were cultured for 3 days with irradiated (3000 rad) syngenic spleen cells as antigen presenting cells (APC) and inactivated MHV-A59 (10^7 plaque-forming units (PFU)/ml). After 3 days, cells were washed and propagated for 7 days in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), streptomycin/penicillin, 2 mM glutamine, 2×10^{-5} M 2-ME (Gibco Laboratories, Grand Island, NY) and 10% Con A-stimulated rat spleen cell supernatant as a source of interleukin-2 (IL-2). Viable cells were harvested on a density gradient (Lympholyte M; Cedarlane Lab., Hornby, Canada), washed intensively and used for transfer experiments.

Adoptive transfer of T-cell lines

BALB/c mice were injected intravenously (i.v.) with 5×10^6 cells of the MHV-A59-specific $CD4^+$ CTL clone HS1, in 100 μl phosphate-buffered saline (PBS). One day later mice were inoculated intraperitoneally (i.p.) with 4000 PFU MHV-A59 ($100 \times LD_{50}$). Control mice received either 5×10^6 PM109 cells, a rabies-specific $CD4^+$ T-cell clone (kindly provided by Dr I. J. T. M. Claassen, RIVM, Bilthoven, the Netherlands) or PBS before MHV-A59 infection. Mice were observed for disease and death once every day for 24 days. To study histology of liver and spleen, tissue virus titres and serum antibody titres, mice were killed 8, 12, 24, 48, 72 and 96 hr post-infection.

Antibodies

The following rat anti-mouse monoclonal antibodies (mAbs) were used for immunohistochemistry: MOMA-1 (IgG2a)[11] and ERTR-9 (IgM)[12] directed against spleen macrophage subpopulations; anti-macrophage mAb MOMA-2 (IgG2b),[13] NLDC-145 (IgG2a)[14] directed against a dendritic cell-associated antigen; 187–1[15] directed against B cells; and Thy-1.2 (Becton Dickinson, Mountain View, CA) directed against T cells. To further characterize T cells, MT4[16] and Lyt2[15] were used to detect $CD4^+$ cells and $CD8^+$ cells, respectively. To detect mouse hepatitis virus antigens, the polyclonal rabbit anti-MHV k134 was used.[17] The unlabelled monoclonal and polyclonal antibodies were used either as undiluted culture supernatant or diluted in PBS containing 0·5% bovine serum albumine (BSA). The enzyme-labelled conjugates, alkaline phosphatase (AP)-conjugated rabbit anti-rat IgG (Sigma, St Louis, MO), peroxidase (PO)-conjugated swine anti-rabbit immunoglobulin and PO-conjugated rabbit anti-rat (both from DAKO, Copenhagen, Denmark) were diluted in PBS/BSA and 1% normal mouse serum (NMS). For enzyme-linked immunosorbent assay (ELISA), the biotinylated antibodies rabbit anti-mouse IgG and rabbit anti-mouse IgM (Zymed Laboratories, San Francisco, CA) and PO-labelled streptavidine (DAKO, Copenhagen, Denmark) were diluted in 10% Protifar milk-powder solution (Nutricia, Zoetermeer, the Netherlands).

Immunohistochemistry

Spleens and livers of mice were aseptically removed and snap-frozen in liquid nitrogen at indicated time points after MHV-A59 infection. Cryostat sections (8 μm) were picked up on gelatin-coated slides, fixed in fresh acetone for 10 min and air dried. Subsequently, slides were horizontally incubated with mAb MOMA-1, MOMA-2, ERTR-9, NLDC-145, 187–1, Thy-1.2, MT4 or Lyt2 for 1 hr at room temperature. Control slides were incubated with PBS/BSA instead of the first specific antibody and examined for non-specific staining. Sections were rinsed three times with PBS. For single immunohistochemical staining, antibodies were visualized with PO-conjugated rabbit anti-rat Ig,[12] using diaminobenzidine-tetra hydrochloride (DAB, Sigma, St Louis, MO) as substrate. Sections were lightly counterstained with haematoxylin, dehydrated and mounted in Entellan (Merck, Germany). Slides that were used for quantification with the IBAS were counterstained with 0·01% nuclear fast red (Merck, Germany) in 5% $Al_2(SO_4)$ in order to discriminate between cells and background. For double immunohistochemical staining, sections were incubated with AP-conjugated rabbit anti-rat IgG for 30 min, followed by staining for AP activity according to Burstone.[18] Subsequently, slides were washed in PBS and incubated with polyclonal rabbit anti-MHV-A59 (k134) for 1 hr. Control slides were incubated with PBS/BSA. After rinsing the slides with PBS (three times), sections were incubated with PO-conjugated swine anti-rabbit immunoglobulin for half an hour and stained for PO activity with 3-amino-9-ethyl-carbazole (AEC, Sigma) according to Graham *et al.*,[19] modified by Claassen *et al.*[20] Finally, the sections were briefly counterstained with haematoxylin, rinsed in tap-water and mounted in Aquamount (BDH Laboratories Supplies, Poole, UK).

Tissue-derived virus titration by end-point dilution assay

MHV-A59 titres in livers and spleens were determined by an end-point dilution assay. Briefly, livers (200 mg/2 ml DMEM) and spleens (50 mg/1 ml DMEM) of MHV-A59-infected mice were aseptically removed at indicated time points after infection, and the tissues were homogenized. L-cells were cultured in 96-wells flat-bottom plates at a density of 3×10^4 cells/well for 24 hr (37°, 5% CO_2) to form monolayers. Serial dilutions of the tissue homogenates were prepared and added to the L-cell monolayer in triplicate. After 3 days, presence of plaques was determined by microscopy. The 50% tissue culture infectious dose values ($TCID_{50}$) were calculated by using the Spearman/Kaerber relationship.[21]

Virus neutralization by MHV-A59-specific antibodies

Sera from MHV-A59-infected mice were tested for their capability to neutralize MHV-A59. Serial dilutions of test sera were incubated with 100 PFU MHV-A59 for 1 hr (37°, 5% CO_2) and added to monolayers of L-cells. The rabbit anti-MHV k134 was

used as a positive control, whereas NMS was used as a negative control. Infection of L-cells was monitored by microscopy after 2 days.

ELISA

Anti-MHV antibodies in serum of infected mice were detected by a direct ELISA. Microtitre 96-wells plates (Nunc, Denmark) were coated for 3 hr at 37° and overnight at 4° with UV-inactivated MHV-A59, 10^5 PFU/well, diluted with coating buffer (0·1 M $NaHCO_3$, pH 9·6). Plates were washed three times with washing buffer (0·05% Tween 20 in PBS) followed by blocking with 10% Protifar solution for 1 hr at 37°. Next, the plates were washed three times and incubated with serial dilutions of test sera (50 μl/well) for 2 hr at 37°. As a control, NMS was used. All samples were tested in duplicate. After washing (three times), either biotinylated rabbit anti-mouse IgM or rabbit anti-mouse IgG was added at 50 μl/well and incubated for 2 hr at 37°. Subsequently, plates were washed and incubated with peroxidase conjugated-streptavidin for 1 hr at 37°. As a substrate, ortho-phenylene-diamine-dihydrochloride (OPD, Sigma) (50 μl/well) with freshly added H_2O_2 was used. The reaction was stopped after 10 min by addition of 1 M H_2SO_4 (25 μl/well). The absorption was measured by ELISA reader (Organon Technika, Oss, the Netherlands) at 492 nm. Serum titres are presented as the highest dilution with an optical density (OD) of 0·05 above negative control serum.

Quantification of T-cell infiltrates

Quantification of the number of infiltrating T cells in liver sections was done using the image analysis system IBAS (Kontron Elektronik, Gmbh, Germany) as described previously.[22,23] Of each liver, 10 microscopic fields (magnification ×10) were studied in three sections. Fields were randomly selected to measure the number of T cells. Results are expressed as mean number of cells ± SD per microscopic field.

RESULTS

Localization of MHV-A59 in liver

To study the route of MHV-A59 infection in spleen and liver, BALB/c mice were i.p. inoculated with 4000 PFU MHV-A59. Localization of MHV-A59 was analysed by a single immunohistochemical staining with polyclonal antibody k134. MHV-A59-infected cells were already present 8 hr post-infection. Double staining with MOMA-2 and k134 revealed that these were MHV-A59-infected macrophages. At 12 hr post-infection, hepatocytes were also infected with MHV-A59 (Fig. 1) and small necrotic foci of inflammation were observed 24 hr after inoculation, and these increased in time. Examination of slides double stained with either MOMA-2, Thy-1.2 or 187-1 and k134 showed that both macrophages and T cells infiltrate the MHV-A59-infected foci. Closer examination of the infiltrated T cells, by staining the sections with either mAb MT4 or Lyt2, revealed that all T cells present were $CD4^+CD8^-$. No B cells were present in the liver. The number of T cells present in the liver was quantificated by IBAS. Figure 2 shows that the number of T cells increases significantly in time.

Localization of MHV-A59 in spleen

To determine the course of MHV-A59 infection in the spleen, single immunohistochemical staining was performed with MHV-specific polyclonal antibody k134 on spleen sections. MHV-A59-infected cells were already detectable in the marginal zone and in the outer periphery of the white pulp of the spleen 12 hr post-infection (Fig. 3A). In time, the virus infects inceasing cell numbers in the marginal zone and infiltrates the white pulp. At 72 hr post-infection, MHV-A59 is also present in cells in the red pulp area of the spleen. To

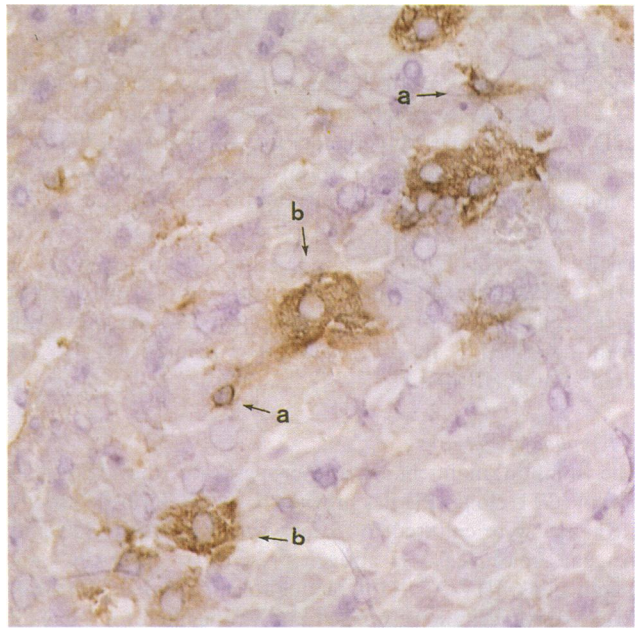

Figure 1. Immunohistochemical staining of liver sections. Livers were removed at different time points after infection and MHV-A59 was detected using single immunohistochemical staining with k134. Shown is a liver section 12 hr after infection, ×10·4. a, k134 positive macrophages, b, k134 positive hepatocytes.

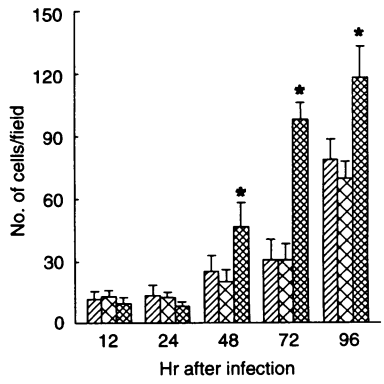

Figure 2. T-cell infiltration of MHV-A59-infected livers. Mice were injected i.v. either with 5×10^6 cells of T cell clone HS1 (▩, $n = 2$) or 5×10^6 cells of T-cell clone PM109 (▩, $n = 1$) or PBS (▨, $n = 1$) as a control. All three groups were challenged (i.p.) after 1 day with 4000 PFU MHV-A59 ($100 \times LD_{50}$). Livers were removed at indicated time points and sections were stained for Thy-1. The number of infiltrating T cells was quantificated using image analysis system IBAS. Shown is one representative out of three performed experiments. Data are expressed as mean number of cells ± SD per microscopic field. *Significant difference $P < 0{\cdot}001$, Student's *t*-test.

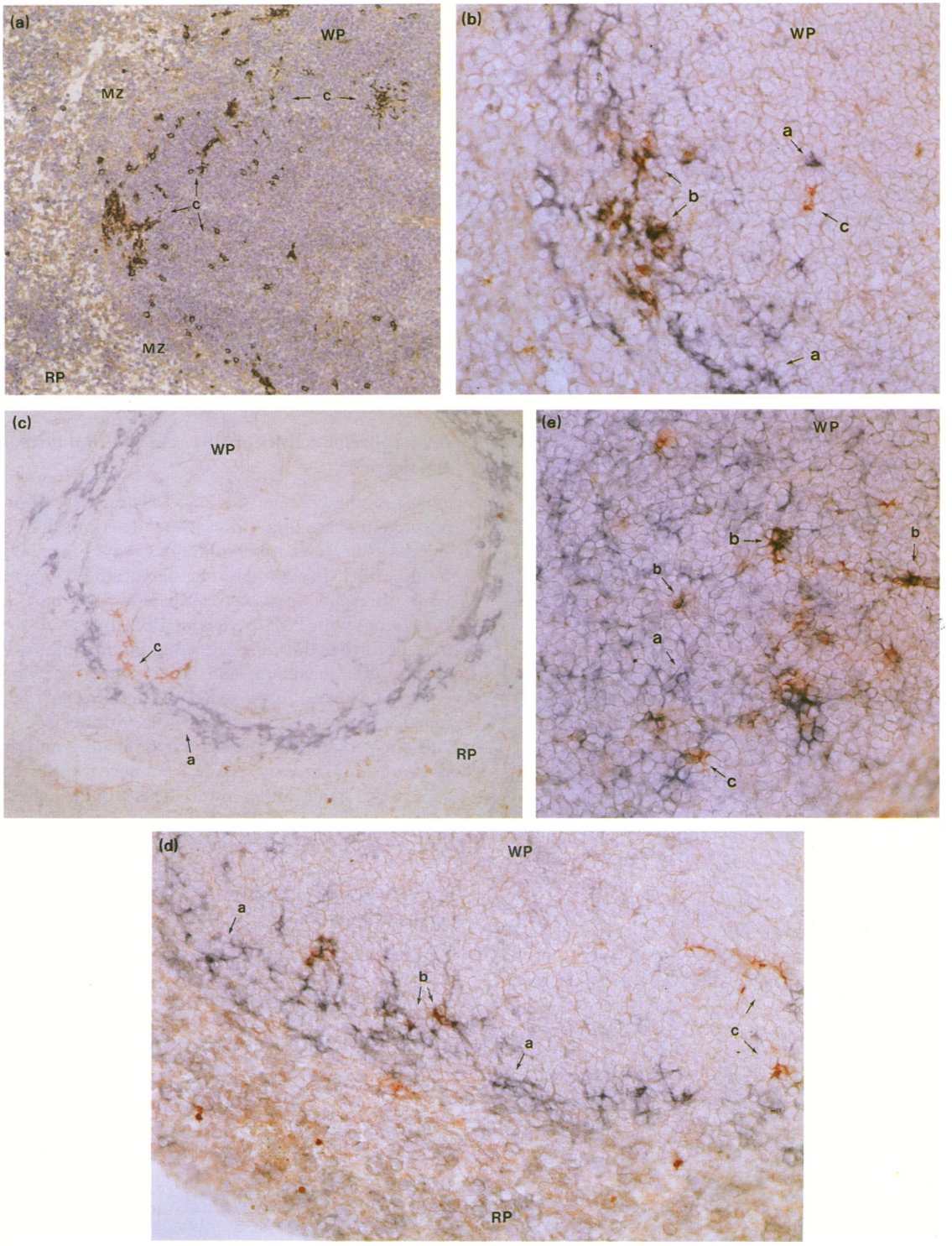

Figure 3. Immunohistochemical staining of spleen sections. Spleens were removed at indicated time points after infection and MHV-A59-infected cells were characterized using double immunohistochemistry. Shown are (a) single staining with k134, 24 hr post-infection, ×10·4; (b) double staining with MOMA-1 and k134, 12 hr post-infection, ×20·8; (c) double staining with ERTR-9 and k134, 12 hr post-infection, ×10·4; (d) double staining with ERTR-9 and k134, 24 hr post-infection, ×20·8; (e) double staining with NLDC-145 and k134, 48 hr post-infection, ×20·8. RP, red pulp; WP, white pulp; MZ, marginal zone; a, cells single positive for either MOMA-1, ERTR-9 or NLDC-145; b, cells double positive for either MOMA-1, ERTR-9 OR NLDC-145 and k134; c, cells single positive for k134.

further characterize the MHV-A59-infected cells, we used monoclonals recognizing different macrophage subsets, dendritic cells, B cells and T cells. The mAb MOMA-2 was used to identify all macrophages present. Indeed, double-positive cells were observed 12 hr post-infection, indicating the presence of MHV-A59 in macrophages. To further characterize which subsets of macrophages were infected, mAbs MOMA-1 and ERTR-9 were used to identify marginal metallophillic macrophages (MMM) and marginal zone macrophages (MZM) respectively. Double staining of spleen sections with MOMA-1 and k134 revealed that 12 hr post-infection MMM were infected (Fig. 3b). In contrast, no cells double positive for ERTR-9 and k134 could be detected (Fig. 3c). Later in infection, more MHV-A59-infected MMM were present and also MZM were infected with MHV-A59 (Fig. 3d). To identify dendritic cells (DC), mAb NLDC-145 was used and it was shown that DC were infected with MHV-A59 48 hr after infection (Fig. 3e). Moreover, double staining of the sections with mAb 187–1 revealed that B cells are also infected with MHV-A59 from this time point. No cells double positive for Thy-1.2 and k134 could be detected during infection, suggesting that T cells are not susceptible to MHV-A59 infection. These data indicate that in the spleen, MHV-A59 first infects the MMM which is followed by infection of the MZM. Then the virus infiltrates into the white pulp, infecting the white pulp macrophages (WPM), DC and B cells. Later in infection, the red pulp macrophages (RPM) are also infected.

Effect of adoptive transfer of T-cell clones on localization of MHV-A59

As described previously, mice infected with MHV-A59 generate a virus-specific $CD4^+$ CTL response. Transfer studies using the MHV-A59-specific $CD4^+$ CTL clone showed a significant protection against a lethal challenge with MHV-A59, implicating that these $CD4^+$ CTL play a pivotal role in the protection against MHV-A59 infections.[8] In this study, we investigated the effect of adoptive transfer of this T-cell clone on localization of MHV-A59. Figure 4 shows that, in contrast to the PM109 cells and the PBS-treated mice, HS1 cells are able to protect mice against a lethal challenge with MHV-A59. Cryostate sections of liver and spleen were double stained for one of the different macrophage, dendritic-cell, T-cell or B-cell markers and for MHV-A59, and the results were compared with those of the control mice. With respect to the first time point after infection at which MHV-A59-infected cells were detected, no differences were observed between the differently treated mice. Moreover, no differences were observed in localization pattern of the virus, either in the liver or in the spleen. In the liver however, the number and size of necrotic areas was diminished. Importantly, liver sections of mice adoptively transferred with HS1 cells showed a significant increase in the number of T cells in the inflamed areas of the livers 48 hr after infection compared with control mice (Fig. 2), with a maximum threefold difference 72 hr after infection. Similar to the control mice, all T cells present in the HS1 T-cell transferred animals were $CD4^+CD8^-$. This indicates that $CD4^-CD8^+$ lymphocytes are not necessary to overcome mouse hepatitis virus infection.

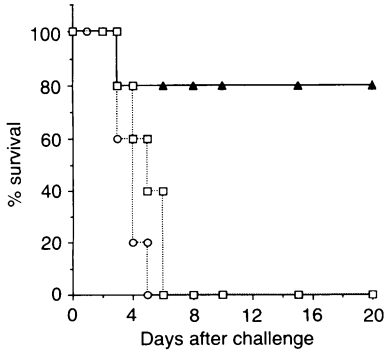

Figure 4. HS1 protects against lethal MHV-A59 challenge. Groups of five mice were injected i.v. either with 5×10^6 cells of T cell clone HS1 (▲, $n = 5$) or 5×10^6 cells of T cell clone PM109 (○, $n = 5$) or PBS (□, $n = 5$) as a control. All three groups were challenged after 1 day with 4000 PFU MHV-A59 ($100 \times LD_{50}$) i.p.. Results are one representative out of three performed experiments.

In vivo effector activity of HS1 cells and viral titres in liver and spleen

To assess the effect of the HS1 cells on virus clearance from infected tissues, viral titres were determined in livers and spleens using an end-point dilution assay. Fig. 5(a) shows that transfer of HS1 cells results in a significant two-log reduction in virus titres in liver as compared with mice adoptively transferred with PM109 cells or PBS. In the HS1 cell-treated mice, a further three-log reduction in virus titre was observed 10 days after infection, and 24 days after infection no virus could be detected in the liver. As can be seen from Fig. 5(b), kinetics of MHV-A59 titres in spleen differ from liver titres. Although spleen virus titres decrease in all groups after 2 days post-infection, titres in spleens from mice transferred with HS1 cells were significantly lower. These data suggest that the MHV-A59-specific $CD4^+$ T cells are involved in clearance of virus from the liver and spleen.

Effect of adoptive transfer of T-cell clones on anti-MHV antibody production

Subsequent to adoptive transfer of the MHV-A59-specific

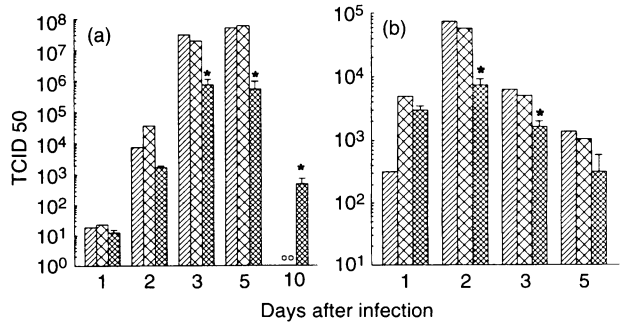

Figure 5. Adoptive transfer of HS1 cells results in decreased viral titres. Mice were injected i.v. either with 5×10^6 cells of T-cell clone HS1 (▩, $n = 2$), 5×10^6 cells of T-cell clone PM109 (▩, $n = 1$) or PBS alone (▨, $n = 1$). All groups were challenged (i.p.) after 1 day with 4000 PFU MHV-A59 ($100 \times LD_{50}$). Viral titres in livers and spleens were determined by end-point dilution assay. Data are expressed as $TCID_{50}$ per 200 mg liver (a) or 50 mg spleen (b). Shown is one representative out of three performed experiments. *Significant difference; ○, mice died.

Table 1. Anti-MHV-A59 antibody production after adoptive transfer of T-cell clones

	^{10}log serum IgM titre		^{10}log serum IgG titre	
T-cell transfer	$d = 4$	$d = 24$	$d = 4$	$d = 24$
–	1·5	*	n.d.	*
PM109	2·0	*	n.d.	*
HS1	2·7	2·2	n.d.	4

Mice were injected i.v. either with 5×10^6 cells of T-cell clone HS1 ($n = 2$) or 5×10^6 cells of T-cell clone PM109 ($n = 1$) or PBS alone ($n = 1$) and 24 hr later challenged with 4000 PFU MHV-A59. Serum IgM and IgG titres were determined by ELISA. Shown are results of one representative out of three performed experiments. *Mice died after lethal MHV-A59 infection; n.d., not detectable.

$CD4^+$ T-cell clone the anti-MHV antibody production was studied. Serum was collected from mice killed at indicated time points after infection and anti-MHV titres were determined by ELISA. Serum IgM titres could be detected from 4 days after infection with MHV-A59. Table 1 shows that serum IgM titres increased marginally after transfer of HS1 cells compared with control mice. Although serum IgG titres were absent early in infection, high anti-MHV IgG titres were detected 24 days post-inoculation in mice that had survived the infection. The virus neutralization assays showed that sera from mice killed 4 days after infection, containing MHV-A59-specific IgM only, were not able to neutralize MHV-A59, whereas sera from mice killed 24 days after infection neutralized the MHV-A59 very well (results not shown).

DISCUSSION

In response to a viral infection, the immune system is designed to evoke a rapid and efficient reaction to eliminate virally infected cells in order to prevent virus-induced damage. Besides the non-specific effector mechanisms, specific reactions of $CD8^+$ CTL, $CD4^+$ Th-1 cells and cytokines produced by these cells are regarded to be of major importance.

In our attempt to reveal the defense mechanism against MHV-A59 infection, we performed an immunohistological study on MHV-A59-infected spleen and liver tissue. Moreover, we studied the effect of adoptive transfer of MHV-A59-specific $CD4^+$ CTL on localization and clearance of virus. Our results show that in the liver, MHV-A59 was first detected in Kupffer cells and later in hepatocytes resulting in severe necrotic foci. In the spleen, infection starts in MMM and MZM and subsequently spreads into WPM, DC and B cells throughout the spleen. Mice transferred with MHV-A59 specific T-cell clone HS1 prior to a lethal challenge with MHV-A59 were able to overcome infection and virus was completely cleared from infected tissues. Importantly, a significant increase in the number of infiltrating $CD4^+$ T lymphocytes was observed in the liver, already early in infection, whereas no effect was found on the localization of MHV-A59, either in the spleen or in liver tissue. Moreover, similar as control mice, no $CD8^+$ T lymphocytes were present in the liver of mice adoptively transferred with HS1 cells. In addition, viral titres in infected tissues were significantly decreased and necrotic foci in the liver were reduced both in size and number. All together, these results imply that $CD4^+$ T cells play an important role in recovery from MHV-A59 infection.

Upon histological examination of tissue sections of infected spleens, we found infection starts in MMM and MZM, and that 48 hr post-infection B cells and DC are also infected. This correlates well with the previously described high expression of MHV-A59 receptors on macrophages and B cells.[24] In the latter study, receptors for MHV-A59 on DC were not described, but this may well be because of the fact that DC were lost during the isolation procedure used to purify macrophages, B cells and T cells.

Macrophages are regarded as important cells with respect to clearance of particulate antigens from a host, because of their scavenging functions and their strategic localization.[25] Blood antigens, such as bacteria or viruses, enter the spleen on the border of white and red pulp, i.e. the marginal zone. Indeed MMM and MZM, present in the outer part of the white pulp and in the marginal zone, respectively, are the first cells infected by MHV-A59. In the liver, macrophages appeared to be first target cells of the virus as well, followed by infection of hepatocytes. The fact that later in infection, DC and B cells are also infected by MHV-A59 suggests that macrophages are not the only cells involved in the induction of a specific cell-mediated immune reponse. The exact role of macrophages in the induction of the CTL response against MHV-A59 infection and clearance of virus from the liver is currently being investigated.

Recovery from viral infections is generally attributed to $CD8^+$ virus-specific CTL.[1,26] Recently, however, studies in several viral systems have shown that $CD4^+$ CTL can play a major role in recovery from viral infections as well. Both in experimental measles encephalitis[3] and experimental influenza virus infection,[2,4] it was shown that $CD4^+$ cells are essential for recovery from virus infection. In addition, also in recovery from coronavirus infections, a role for $CD4^+$ T cells has been described,[27–29] although some controversy exists concerning their role in clearance of infectious virus. For instance, Stohlman *et al.*[27] showed that transfer of $L3T4^+$ T-cell clones protected against lethal infection by MHV-JHM, though they did not observe suppression of viral replication. Moreover, it was shown that MHV-JHM-specific $CD8^+$ T cells have to cooperate with $CD4^+$ T cells to eliminate virus.[30] In contrast, Korner *et al.*[29] did observe clearance of virus from the central nervous system after transfer of $CD4^+$ virus-specific T-cell lines in rats.

The present results demonstrate a major role for virus-specific $CD4^+$ CTL in recovery from MHV-A59 infection in the absence of $CD8^+$ T cells. We observed a significant increase in number of $CD4^+$ T cells infiltrating the infected liver in mice adoptively transferred with HS1 cells, whereas $CD8^+$ T cells were not detectable. Importantly, this increase started 2 days earlier in infection compared with control mice, suggesting that because of the presence of MHV-A59-specific T cells, specific mechanisms function early in infection which results in clearance of virus. In order to determine if the T-cell infiltrates consisted mainly of the transferred HS1 cells, we injected fluorescein isothiocyanate (FITC)-labelled HS1 cells. The same results were obtained with respect to recovery from MHV infection and elimination of infectious virus, indicating FITC-labelling does not affect the function of HS1 cells. However, we were not able to detect FITC-labelled cells,

probably bcause of loss of the FITC label during proliferation and in time (results not shown).

The protective effect of the HS1 cells is associated with the specific elimination of infectious virus from the liver and spleen of recovering animals. Though we observed differences in kinetics between spleen and liver virus titres, the virus was efficiently eliminated from both organs. Since it is already shown that HS1 cells are able to kill MHV-A59-infected cells *in vitro*[8,9] and we noticed the rapid influx of T cells into the infected liver, we favour the possibility that HS1 cells lyse the MHV-A59-infected cells in tissues, resulting in decreased viral titres. In addition to direct cytolysis of MHV-A59-infected cells, recovery from infection may be promoted by antigen-dependent release of cytokines with antiviral activity. *In vitro* results showed that HS1 cells produce IL-2 and IFN-γ when stimulated with MHV-A59-infected syngenic spleen cells.[8,9] IFN-γ has been shown to have multiple functions in host responses to viral infections. Chung *et al.*[28] have shown that addition of IFN-γ to MHV-3-infected macrophage cultures inhibits viral replication. In addition, macrophage effector functions can be induced by IFN-γ and TNF-α,[31,32] resulting in killing of intracellular parasites or viruses and production of IFN-γ by the macrophage itself. Though it has been described that in experimental influenza infection, IFN-γ may not be essential for the antiviral activity of $CD4^+$ T cells,[33] we cannot exclude the possibility that infiltrating HS1 cells which recognize MHV-A59 antigens may indirectly contribute to virus clearance by production of IFN-γ.

Apart from direct killing of MHV-A59-infected cells, the HS1 cells may also promote recovery from infection by providing B-cell help for the production of neutralizing antibodies. Though we were not able to detect anti-MHV-A59 IgG titres early in infection, we did observe a slight increase of virus-specific IgM production in mice that received the HS1 cells. Moreover, these IgM antibodies were not virus neutralizing, and therefore, IgM antibodies are not important for protection. Only in mice that survived the MHV-A59 infection, were high titres of virus-neutralizing serum IgG detected, suggesting these IgG antibodies are probably not involved in the protection mediated by transfer of antiviral $CD4^+$ CTL at early stages of infection.

Previous reports describe natural cytotoxicity in non-immune mice against MHV-A59-infected target cells, mediated by cells with a B-lymphocyte phenotype.[34,35] However, we were not able to detect any B cells infiltrating the infected areas in the liver and therefore conclude that *in vivo*, this B-cell-mediated cytotoxicity is not involved in clearance of MHV-A59 from the liver.

In conclusion, our data suggest that during infection with MHV-A59, $CD4^+$ cytotoxic T cells play a crucial role in virus elimination and in recovery from infection, either by direct lysis of MHV-A59-infected cells or indirectly via cytokine production resulting in macrophage activation. Future experiments will focus on the role of macrophages in the non-specific immune response and their function in the induction of the MHV-A59-specific cell-mediated immune response.

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