Mouse hepatitis viral infection induces an extrathymic differentiation of the specific intrahepatic $\alpha\beta$-TCR intermediate LFA-1$^{\text{high}}$ T-cell population

L. LAMONTAGNE, E. MASSICOTTE & C. PAGE  Département des Sciences Biologiques, Université du Québec à Montréal, Canada

**SUMMARY**

Mouse hepatitis virus type 3 (MHV3), a coronavirus, is an excellent model for the study of thymic and extrathymic T-cell subpopulation disorders induced during viral hepatitis. It was recently reported that, in addition to the intrathymic T-cell differentiation pathway, an extrathymic differentiation pathway of $\alpha\beta$-T-cell receptor (TCR) T lymphocytes exists in the liver, and becomes important under pathological situations such as autoimmune diseases, malignancies or hepatic bacterial infections. In the present study, we compared the phenotypes of resident hepatic, splenic or thymic T-cell subpopulations during the acute viral hepatitis induced by MHV3 in susceptible C57BL/6 mice. The number of liver-resident mononuclear cells (MNC) increased during the viral infection, while cellularity decreased. Single positive (SP) CD4$^+$ cells strongly increased in both the liver and thymus, while double positive (DP) (CD4$^+$CD8$^+$) cells, present in the liver and thymus of mock-infected mice, decreased in C57BL/6 mice during the viral infection. A shift of $\alpha\beta$-TCR$^{\text{int}}$ T cells toward $\alpha\beta$-TCR$^{\text{high}}$ was evidenced in the liver and thymus of infected mice, but not in the spleen. The few $\alpha\beta$-TCR$^{\text{int}}$ double negative (DN) (CD4$^-$CD8$^-$) cells also decreased following viral infection. $\alpha\beta$-TCR$^{\text{int}}$ or $^{\text{high}}$ lymphocytes expressing high levels of leucocyte function antigen-1 (LFA-1) increased in the liver of MHV3-infected mice. In addition, liver-resident T cells expressed strongly the CD44 (Pgp-1) activation marker, suggesting that they were either activated or antigen experienced during the viral infection. No significant change in T-cell subpopulations was detected in the spleen, suggesting that MHV3 infection could induce an early in situ differentiation of resident hepatic T cells rather than a recruitment of lymphocytes from peripheral lymphoid organs.

**INTRODUCTION**

T cells play a critical role in the control and elimination of viral infection. Cellular immune responses, mediated by CD4$^+$ and CD8$^+$ T lymphocytes, are dependent upon the thymus for normal T lymphopoiesis and on the integrity of peripheral lymphoid organs for lymphocyte activation. T lymphocytes have been assumed to play an essential role in the of the host immune response to tissue injury in patients with acute or chronic viral hepatitis, but the analysis of mononuclear cells from the peripheral blood of these patients led to controversial results. It was reported that CD4/CD8 ratios of liver-derived lymphocytes from hepatitis B or C patients correlated with viral replication, but not with the CD4/CD8 ratios of peripheral blood lymphocytes, thereby suggesting an in situ regulation of the immune cellular responses.

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Correspondence: Dr L. Lamontagne, Dépt. Sciences Biologiques, Université du Québec à Montréal, C.P. 8888 Succ. A, Centre-Ville, Montréal, Québec, Canada H3C 3P8.

Mouse hepatitis virus type 3 (MHV3), a coronavirus, is an excellent animal model for the study of hepatic T-cell subpopulations during the virus-induced hepatitis. MHV serotypes induce various pathologies as a function of the viral strain, route of infection, and mouse age and strain. C57BL/6 mice, susceptible to MHV3 infection, develop an acute hepatitis and die within 3 days, whereas resistant A/J mice develop only a subclinical infection, with viral clearance occurring within 7 days p.i. (C57BL/6×A/J) F1 mice, in contrast, can survive the acute phase of the hepatitis, and develop a chronic wasting syndrome associated with viral persistence in the liver, brain and lymphoid organs, suggesting a disorder in the immune-mediated viral elimination processes. MHV3 is the most hepatotropic serotype as, in these mouse strains, the liver is the main target organ and the privileged site for viral replication. Numerous necrotic foci and inflammatory mononuclear cells (MNC) can be observed in the liver of infected susceptible C57BL/6 mice, whereas cellular liver lesions remain scarce in resistant mice. Several factors are involved in the pathogenic process of hepatitis. Viral replication in various hepatic cells, such as Kupffer cells, endothelial cells, and hepatocytes, or
disorders in hepatic microcirculation induced by macrophage/monocyte procoagulant activity (PCA) under T-cell control\(^{16-18}\) have been proposed to explain the occurrence and extension of the hepatic lesions. We have previously reported the occurrence of lesions and cellular disorders in lymphoid organs, such as the thymus, spleen, and bone marrow of susceptible C57BL/6 mice acutely infected with MHV3.\(^{12,19,20}\) Resistant mice, however, show no extensive histopathological lesions or cell deficiencies in the lymphoid organs, suggesting a correlation between hepatitis and immune disorders.\(^{12,19,20}\) The thymic disorder, characterized by thymic atrophy and depletion of T-cell subpopulations results from the contact of thymocytes with MHV3-infected thymic stromal cells.\(^{20}\)

Splenic disorders are less evident, suggesting a local interaction between infectious viruses and immune cells, rather than a systemic immune process. No information, however, is available concerning the resident T-cell subpopulations in the liver of MHV3-infected mice, or on their role in the hepatic pathogenic process.

Recent investigations have shown that the liver of normal adult mice contains both \(\alpha\beta\)-T-cell receptor (TCR) and \(\gamma\delta\)-TCR cells, residing in hepatic sinusoids, which appear to be distinct from \(\alpha\beta\)-TCR and \(\gamma\delta\)-TCR T cells found in other peripheral lymphoid tissues.\(^{21-23}\) These T cells are characterized by an \(\alpha\beta\)-TCR of intermediate intensity (\(\alpha\beta\)-TCR\(^{\text{int}}\)), and consist of double-negative (DN) CD4\(^+\) CD8\(^−\) and single-positive (SP) CD4\(^+\) or CD8\(^+\) cells.\(^{22,23}\) The percentage of SP CD4\(^+\) cells is reported to be higher in the liver than in the spleen or thymus.\(^{23,24}\) It was suggested that liver-resident \(\alpha\beta\)-TCR\(^{\text{int}}\) cells are generated as primitive T cells in the digestive tract and subsequently differentiate in the liver.\(^{24}\) Although the hepatic pathway of T-cell differentiation is relatively minor in normal young mice, it becomes more important under pathological situations, such as in autoimmune diseases, malignancy or hepatic bacterial infections.\(^{23,25-27}\) Hepatic T cells have several other unique properties, including the generation of T-cell oligoclonal after bacterial stimulation, and a higher intensity of leucocyte function antigen-1 (LFA-1).\(^{23,27}\) We have no information concerning the role of \(\alpha\beta\)-TCR\(^{\text{int}}\) cell subpopulations during a viral-induced hepatitis.

In the present study, we analysed the phenotypes of resident hepatic, splenic and thymic T-cell subpopulations during the acute viral hepatitis induced by MHV3 in susceptible C57BL/6 mice. In contrast to that seen in thymus or spleen, the number of hepatic MNC actually increased during the viral infection. Hepatic \(\alpha\beta\)-TCR\(^{\text{low or int}}\) cells are shifted to \(\alpha\beta\)-TCR\(^{\text{int or high}}\) T cells in infected mice. In addition, the specific hepatic \(\alpha\beta\)-TCR\(^{\text{int}}\), LFA-1\(^{\text{high}}\) cell population increased in the liver of MHV3-infected mice and remained high in hepatic and thymic \(\alpha\beta\)-TCR\(^{\text{int or high}}\) T cells. Studies with activation markers suggest that hepatic \(\alpha\beta\)-TCR\(^{\text{int}}\) cells may be activated in situ during the viral hepatitis.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were tested, before use, for the presence of anti-MHV antibodies by an enzyme-linked immunosorbent assay (ELISA) test, using a MHV3 preparation as antigen. During the experiments, the animals were housed in a sterile atmosphere (Forma Scientific, Marietta, OH). Female mice between 8- and 12-weeks old were used in all experiments. Mice were anaesthetized with ketamine-sulphate (200 mg/kg) and xylazine (10 mg/kg) by i.p. injection. Mice were bled by section of portal vein and aortic artery, as described by Watanabe et al.\(^{23}\) Liver, spleen and thymus were removed from dead mice after total bleeding.

**Virus**

Pathogenic MHV3 was a cloned substrain produced in L2 cells as previously described.\(^{28}\) Virus was passaged in L2 cells before use and verified regularly as to its pathogenic properties.

**Experimental infections**

Groups of three mice were infected i.p. with 1000 TCID\(_{50}\) (tissue culture infective dose 50%) of pathogenic MHV3, and killed after 72 hr p.i. Mock-infected mice received i.p. a similar volume of phosphate-buffered saline (PBS). Mice were anaesthetized, bled and the organs were collected at 72 hr p.i. Thymuses and spleens were collected, but the hepatic lymphoid cells were enriched by a double Percoll gradient. All cell preparations were electronically counted.

**Cells**

L2 cells, a continuous mouse fibroblast cell line, were grown in Dulbecco’s modified Eagle’s medium (MEM) with glutamine (2 mm), 5% fetal calf serum (FCS), and antibiotics (penicillin, 100U/ml, and streptomycin, 100 mg/ml) (Gibco Laboratories, Grand Island, NY). L2 cells were used for virus propagating, cloning and titrations.

Thymic or splenic cells were obtained from mice bled as described above. The thymus and spleen were collected and pressed through a 70-\(\mu\)m cell strainer (Becton-Dickinson, Lincoln Park, NJ) in RPMI with 20% FCS at room temperature. Cell preparations were electronically counted (Coulter Counter, Coulter Electronics, Hialeah, FL), and cell viability was assayed by the trypan blue exclusion test, ranging from 90 to 100%.

To obtain MNC from the liver, groups of three livers were pressed through a 70-\(\mu\)m cell strainer, which was then washed with 20 ml of RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 20% SFV and antibiotics. The suspension was then underlaid with 5 ml of FCS to allow debris sedimentation. The top layer was then centrifuged on top of a discontinuous Percoll gradient (45%, 67% Percoll in PBS) for 1 hr at 2600 r.p.m. MNC were collected at the interface of the 45% and 67% Percoll layers. The cells were then washed in RPMI-1640 containing 20% FCS and electronically counted as described above.

**Immunolabellings**

**Cytfluorometry.** Double immunolabelling was performed as follows: 10\(^3\) cells were resuspended in 500 \(\mu\)l of RPMI-1640 containing 20% FCS, and incubated on ice for 1 hr with the optimal dilution of monoclonal antibody (mAb) anti-mouse CD4–fluorescein isothiocyanate (FITC) (clone RM-4) (Pharmingen, San Diego, CA), and mAb anti-mouse CD8–phycoerythrin (PE) (clone 53-6.7) (Pharmingen). Cells were then washed in PBS, resuspended in ice-cold 1% formaldehyde in PBS (Fisher Scientific, Montréal, Canada), and fixed overnight. Procedure was similar for CD4/\(\alpha\beta\)-TCR (mAb
anti-mouse zl-TCR–PE: clone H57-597, Pharmingen), LFA-1/zj-TCR (mAb anti-mouse CD11a–FITC: clone M17/4, Pharmingen), CD4+ CD8/zj-TCR, CD11b/zj-TCR (mAb anti-mouse CD11b–FITC: clone M1/70, Pharmingen), B220 (mAb anti-B220–FITC: clone RA3-6B2, Gibco)/TCR, CD44 (mAb anti-mouse CD44–PE: clone IM7, Pharmingen)/zj-TCR, and 45 chains (polycyonal goat anti-mouse 45 chains–FITC) (Caltag Laboratories Inc., San Francisco, CA)/NK1.1 (mAb anti-mouse NK1.1, Pharmingen) double labellings.

The presence of apoptosis in hepatic MNC cells was determined by phenotypic staining and DNA cell content analysis using a modification of technique of Garvy et al.24 Cells labelled with FITC-conjugated anti-zj-TCR, anti-B220 or anti-CD44 antibodies were fixed overnight in 0·1% formaldehyde at 4°C, washed twice in PBS containing 0·005% saponin (Sigma, St Louis, MO) to remove the fixative, and stained in 1 ml of a propidium iodide (PI) staining reagent (PBS pH 7·4 with 0·05 mg/ml RNase at 50 U/ml and 50 μg/ml PI) at 4°C. Cytophuorometric analysis was done on a fluorescence-activated cell sorting (FacScan) cytofluorometer with Lysis II Software (Becton-Dickinson). Gating was performed according to forward scatter versus 90° angle scatter (side scatter) so as to select the distinct mononuclear cell populations. Five thousand cells selected from this gate were analysed.

**Fluorescence microscopy.** Immunolabelling of intracellular viral proteins was carried out in *in vivo* infected hepatic MNC. Samples of 4 × 10⁵ cells were deposited on glass slides previously coated with 100 μl PBS/bovine serum albumin (BSA) (Gibco), and cytocentrifuged (Cytospin; Shandon, Southern Instrument Inc., Sewickly, PA) at 1000 r.p.m. for 5 min. The samples were air-dried, then soaked in precooled acetic acid–ethanol (5%/v/v) for 12 min on ice, and washed five times in cooled PBS. The cytospots were stained with an optimal dilution of polyclonal anti-MHV3 antisera from immunized A/J mice (ELISA titre 1:2048), incubated for 1 hr at room temperature in a humidified chamber, washed four times with PBS, treated with FITC rabbit anti-mouse Fab (immunglobulin G: IgG) (Dimension Laboratories, Mississauga, Ontario, Canada), incubated for an additional 30 min, and rewatched in PBS. Double immunolabelling using mAb anti-Mac-1–FITC (clone M1/70; Pharmingen), in conjunction with polyclonal anti-MHV3 antisera and TRITC rabbit anti-mouse Fab (IgG) was also performed. Slides were mounted with a medium containing 90% glycerol (Fisher Scientific Co., Montréal, Québec, Canada) in PBS (pH 8·0) and 0·1% p-phenylenediamine (Fisher Scientific Co.). A fluorescence microscope (Leitz Dialux 22, Midland, Ontario, Canada), equipped with a mercury lamp and phase contrast optics, was used for observation.

**Virus titration**

Hepatic MNC, splenic or thymic cell preparations were frozen at −70°C, thawed, and then centrifuged, with the supernatants used as viral suspensions. These were then serially diluted in 10-fold steps in Dulbecco’s MEM and tested on L2-cells cultured in 96-well microtitre plates. Cytopathic effects (CPE), characterized by syncytia formation and cell lysis, were recorded at 72 hr p.i. and virus titres expressed as log₁₀ 50% TCID₅₀. All titrations were made in triplicate.

**Statistical analysis**

Percentage and absolute numbers were evaluated by Student’s t-test. The ratios were analysed by the Wilcoxon–Mann–Whitney U-test.

**RESULTS**

**Number of hepatic mononuclear cells, and total cellularity of spleen, and thymus of C57BL/6 mice infected with MHV3**

We have previously demonstrated a correlation between cell depletion in thymus or bone marrow and the susceptibility to acute hepatitis in C57BL/6 mice.12,19,20 To verify if hepatitis outcome was associated with an intrahepatic immunodeficiency in spite of the presence of inflammatory foci in the liver of infected mice, susceptible C57BL/6 mice were i.p. injected with 1000 TCID₅₀ of MHV3 virus (10 TCID₅₀ is equivalent to one LD₅₀). Mock-infected mice received i.p. a similar volume of PBS. Mice were anaesthetized, bled, and the liver, spleen, and thymus were collected at 72 hr p.i. As shown in Table 1, the number of liver-resident MNC increased in MHV3-infected C57BL/6 mice. The number of cells isolated from the liver, however, is about 10 times less than from in lymphoid organs. No significant decreases were detected in spleen cellularity, whereas thymic cells were strongly depleted (P < 0·001). These observations suggest that hepatic MNC may be stimulated *in situ* rather than recruited from peripheral immune organs during viral hepatitis.

**Morphological analysis, after Giemsa staining, of liver- and spleen-resident MNC suspensions from mock-infected C57BL/6 mice revealed that MNC represent more than 90% of the cells. Few macrophages (less than 3%) (CD11b+), B, and natural killer (NK) cells (less than about 5%) (NK1.1+) were found in liver MNC preparations.**

**Analysis of the CD4–CD8 phenotypes of hepatic, splenic and thymic T lymphocytes isolated from C57BL/6 mice infected with MHV3**

It was previously observed, in humans and mice, that percentages of hepatic T-cell subpopulations differed from those of lymphoid organs.5,6,23,24 In addition, mature T-cell generation can also occur extrathymically, particularly in the liver.21–23,25 To determine if the increase in hepatic MNC cells induced by MHV3 infection results from a stimulation of specific T-cell subpopulations, the CD4–CD8 phenotypes of liver-resident MNC from viral-infected or mock-infected C57BL/6 mice were analysed and compared with those of splenic and thymic lymphocytes.

**Table 1. Number of hepatic, splenic and thymic mononuclear cells from C57BL/6 mice infected with MHV3**

| Virus | Liver | Spleen | Thymus |
|-------|-------|--------|--------|
| Uninfected | 2.1 ± 0.4 | 57.9 ± 5.5 | 83.7 ± 8.7 |
| MHV3 | 4.3 ± 0.8† | 58.8 ± 8.2 | 23.3 ± 10.9† |

*Data are means ± SD of three mice per group. These results are representative of three different experiments.

†P < 0·001.
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Table 2. Percentages of CD4–CD8 phenotypes of hepatic, splenic and thymic T lymphocytes isolated from C57BL/6 infected with MHV3

| Organ | Virus | CD4+ CD8– | CD4− CD8+ | CD4+ CD8+ | CD4/CD8 ratio |
|-------|-------|-----------|-----------|-----------|---------------|
| Liver | Uninfected | 18·9 ± 1·7 | 4·7 ± 1·5 | 1·4 ± 0·2 | 4·1 ± 0·9 |
|       | MHV3   | 30·6 ± 1·4† | 6·2 ± 0·1‡ | 0·4 ± 0·2† | 4·9 ± 0·3 |
| Spleen| Uninfected | 15·9 ± 2·3 | 6·1 ± 2·3 | NA         | 2·6 ± 0·1 |
|       | MHV3   | 15·8 ± 3·5 | 4·3 ± 1·8 | NA         | 2·3 ± 0·1‡ |
| Thymus| Uninfected | 9·7 ± 2·4 | 0·7 ± 0·3 | 88·8 ± 2·6 | NA           |
|       | MHV3   | 26·1 ± 4·8† | 2·7 ± 1·3 | 69·5 ± 3·2† | NA           |

*The cell preparations were double-labelled with FITC anti-CD4 mAb, and PE anti-CD8 mAb, and analysed by flow cytometry. Data are means ± SD of three mice per group. These results are representative of three different experiments.
†P < 0·001.
‡P < 0·01.
NA not applicable.

cell preparations. As shown in Table 2 and Fig. 1, percentages of both SP CD4+ (P < 0·001) and CD8+ (P < 0·01) cells increased in the liver from MHV3-infected C57BL/6 mice. Splenic SP CD4+ or CD8+ T-cell subpopulations were not altered whereas percentages of SP CD4+ cells in thymus strongly increased in infected animals (P < 0·001). We observed an abrupt decrease in thymic double positive (DP) cells (P < 0·001) (Table 2). Similarly, the small hepatic-resident DP cells decreased also sharply (P < 0·001) (Table 2). It was recently reported that, in mice, the higher hepatic CD4/CD8 ratio was due to a greater number of CD4+ T cells. This was confirmed in mock-infected C57BL/6 mice (Table 2). In MHV3-infected animals, the CD4/CD8 ratio was maintained although the percentages of both CD4+ and CD8+ cells were higher. Moreover, viral infection slightly alters the CD4/CD8 ratio of splenic cells in infected C57BL/6 mice.

αβ-TCR/CD4–CD8 profile patterns of hepatic, splenic and thymic T lymphocytes isolated from C57BL/6 mice infected with MHV3

It has been previously shown that αβ-TCRint cells, normally only present in thymus, were also found among the liver-resident MNC suggesting an extrathymic differentiation pathway. Similarly, the small hepatic-resident DP cells decreased also sharply (P < 0·001) (Table 2). It was recently reported that, in mice, the higher hepatic CD4/CD8 ratio was due to a greater number of CD4+ T cells. This was confirmed in mock-infected C57BL/6 mice (Table 2). In MHV3-infected animals, the CD4/CD8 ratio was maintained although the percentages of both CD4+ and CD8+ cells were higher. Moreover, viral infection slightly alters the CD4/CD8 ratio of splenic cells in infected C57BL/6 mice.

![Figure 1. Phenotypic characterization with CD4, and CD8 antigens of hepatic MNC (a and d), splenic (b and e) or thymic (c and f) cells from mock-infected (a,b, and c) or MHV3-infected (d,e, and f) C57BL/6 mice. The cell preparations were double-labelled with FITC anti-CD4 mAb, and PE anti-CD8 mAb, and analysed by flow cytometry. Numbers indicate the percentage of fluorescence-positive cells in the corresponding squares. These results are representative of three different experiments.](image)
a shift toward $\alpha$$\beta$-TCR$^{\text{high}}$ T cells in hepatic and thymic MNC from MHV3 infected C57BL/6 mice, as evidenced by decreases in $\alpha$$\beta$-TCR$^{\text{low}}$ and $\alpha$$\beta$-TCR$^{\text{int}}$ cells, and by an increase in $\alpha$$\beta$-TCR$^{\text{high}}$ cells (Fig. 2a,c,d,f). This shift was particularly evident in the hepatic cell preparation. No significant shift was detected in splenic T-cell populations (Fig. 2b,e).

In addition, double labelling for CD4 and $\alpha$$\beta$-TCR revealed that percentages of hepatic and thymic $\alpha$$\beta$-TCR$^{\text{high}}$ CD4$^+$ cells in total lymphoid cells increased strongly in MHV3-infected C57BL/6 mice ($P<0.001$), whereas thymic $\alpha$$\beta$-TCR$^{\text{low}}$ CD4$^+$ cells decreased ($P<0.001$; Table 3). All CD4 cell subpopulations decreased in the spleen of infected C57BL/6 mice ($P<0.01$ to $P<0.001$). Comparable results were also observed with hepatic, splenic and thymic SP CD8$^+$ cells (results not shown). The increase of $\alpha$$\beta$-TCR$^{\text{high}}$ CD4$^+$ cells as seen in the liver, but not in the spleen, of infected mice is compatible with a differentiation pathway stimulated in situ during the viral infection, but not with a recruitment of splenic antigen-activated T cells. The percentage increase in thymic $\alpha$$\beta$-TCR$^{\text{high}}$ CD4$^+$ cells in MHV3-infected C57BL/6 mice can reflect the higher loss of $\alpha$$\beta$-TCR$^{\text{low}}$ DP cells in the depleted thymus rather than a stimulation of T-cell differentiation pathway since the absolute number of the T-cell subpopulation did not increase in atrophic thymus.

A specific liver-resident DN $\alpha$$\beta$-TCR$^{\text{int}}$ cell population has also been reported. If the viral infection stimulates an intrahepatic immature T-cell differentiation pathway, $\alpha$$\beta$-TCR$^{\text{int}}$ or $\text{high}$ DN cell levels should decrease. To test this hypothesis, we analysed the $\alpha$$\beta$-TCR profile patterns of DN cells from the liver of MHV3 C57BL/6 infected mice (Fig. 3). As expected, a shift from DN to SP CD4$^+$ or and CD8$^+$ cells was shown by a percentage decrease of hepatic $\alpha$$\beta$-TCR$^{\text{int}}$ or $\text{high}$ DN cells, as well as an increase of SP CD4$^+$ and/or CD8$^+$ cells. In the thymus, few $\alpha$$\beta$-TCR$^+$ DN cells were also detected. Percentages of $\alpha$$\beta$-TCR$^{\text{int}}$ or $\text{high}$ DN thymic cells increased slightly in MHV3-infected C57BL/6 mice ($\alpha$$\beta$-TCR$^{\text{int}}$ 0.02 ± 0.01–0.19 ± 0.06; $\alpha$$\beta$-TCR$^{\text{high}}$ 0.07 ± 0.02–0.21 ± 0.05) ($P<0.001$), reflecting the important decrease in cells in the atrophic thymus rather than an increase in the number of such DN cells.

### Table 3. TCR-$\alpha$$\beta$ expression levels in CD4$^+$ lymphocytes isolated from liver, spleen and thymus from C57BL/6 mice at 72 h after infection with MHV3

| Organ | Virus | TCR$^{\text{low}}$ | TCR$^{\text{int}}$ | TCR$^{\text{high}}$ $^*$ |
|-------|-------|-------------------|-------------------|---------------------|
| Liver | Uninfected | 0.35 ± 0.02 | 3.53 ± 0.91 | 6.21 ± 1.23 |
|       | MHV3   | 0.25 ± 0.09 | 8.42 ± 4.81 | 20.5 ± 2.72 $^*$ |
| Spleen| Uninfected | 0.44 ± 0.09 | 1.06 ± 0.26 | 15.5 ± 0.28 |
|       | MHV3   | 0.12 ± 0.03 $^+$ | 0.64 ± 0.34 | 12.3 ± 0.45 $^+$ |
| Thymus| Uninfected | 59.4 ± 0.29 | 30.1 ± 0.88 | 11.0 ± 0.58 |
|       | MHV3   | 25.6 ± 8.2 | 34.9 ± 2.90 | 35.9 ± 4.71 $^+$ |

$^*$The cell preparations were double-labelled with FITC anti-CD4 mAb, and PE anti-TCR mAb, and analysed by flow cytometry. Data are means ± SD of three mice per group. These results are representative of three different experiments.

$^+$P < 0.001.

$^*$P < 0.01.
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Figure 3. Phenotypic characterization with CD4 and CD8, and zβ-TCR antigens of hepatic MNC from mock-infected (a) or L2-MHV3-infected (b) C57BL/6 mice. The cell preparations were double-labelled with FITC anti-CD4 mixed with FITC anti-CD8 mAbs, and PE anti-zβ-TCR mAb, and analysed by flow cytometry. These results are representative of three different experiments.

Table 4. TCR-zβ expression levels in LFA-1high lymphocytes isolated from liver, spleen and thymus from C57BL/6 mice at 72 hr after infection with MHV3

| Organ     | Virus  | TCRint | TCRhigh | TCRbhigh |
|-----------|--------|--------|---------|-----------|
| Liver     | Uninfected | 14.6±1.1 | 6.3±2.9 | 15.2±2.9 |
|           | MHV3   | 15.4±3.4 | 12.5±1.2† | 27.1±6.5† |
| Spleen    | Uninfected | 53.3±7.9 | 23±0.9 | 104±3.5 |
|           | MHV3   | 42.4±18.8 | 44±2.7 | 170±8.7 |
| Thymus    | Uninfected | 73.7±9.5 | 25.4±9.6 | 2.4±0.3 |
|           | MHV3   | 40.7±13.5† | 45.5±6.8† | 164±3.9† |

*The cell preparations were double-labelled with FITC anti-LFA-1 mAb, and PE anti-zβ-TCR mAb, and analysed by flow cytometry. Data are means±SD of three mice per group. These results are representative of three different experiments.
†P<0.001.

Profile patterns of CD44 (Pgp-1) activation marker in hepatic, splenic and thymic T lymphocytes isolated from MHV3-infected C57BL/6 mice

To participate in the immune-mediated viral elimination process, SP CD4+ or CD8+ T cells have to be activated. It was previously reported that many hepatic zβ-TCRint express IL-2Rβ and CD44 (Pgp-1) cell surface markers, as seen on antigen-experienced T cells, suggesting that these cells may be activated in situ. To determine if resident hepatic T lymphocytes from infected mice can participate in immune responses, we analysed the CD44 expression profile patterns of hepatic MNC from the liver of mock-infected and MHV3-infected C57BL/6 mice. As shown in Fig. 4(a), higher number of CD44high cells was found in hepatic MNC than in spleen or thymus from mock-infected mice (Fig. 4b,c). CD44high cells strongly increased in liver preparations from MHV3-infected mice (Fig. 4d), but not in the spleen (Fig. 4e). However, CD44 expression level increased slightly in thymocytes from MHV3-infected mice (Fig. 4f). Double staining for TCR and CD44 markers indicated that the hepatic TCRintCD44high cell population in liver increased strongly in MHV3-infected mice (12.89±3.52–20.30±2.25%) (P<0.001), but not in spleen or thymus (results not shown). These results suggest that activated T cells are generated in situ in the liver during viral infection.

Levels of apoptotic cells in hepatic, splenic and thymic T lymphocytes isolated from MHV3-infected C57BL/6 mice

A recent study suggested that the liver could be involved in a general clearance pathway of mature peripheral T cells following TCR ligation by antigens. Such activated cells express the CD45R (B220) marker and accumulate in the liver wherein Table 4, in mock-infected mice, the percentage of B220high cells among total lymphoid cells increased slightly in hepatic MNC or thymic cells from MHV3-infected C57BL/6 mice. As expected, thymocyte B220 expression remained low, despite the slight increase with infected mice. PI staining was used to verify the presence of hypoploid cells in hepatic MNC or thymic cells from MHV3-infected C57BL/6 mice. As shown in Table 5, percentages of B220 high T cells was also seen in thymic cells from MHV3-infected mice (P<0.001: Table 4). These results suggest that MHV3 infection induces an increase of zβ-TCRint or high LFA-1high T cells in the liver, and to a lesser extent, in the thymus.
Figure 4. A comparison of expression profiles (single-colour staining of CD44) of hepatic MNC (a and d), splenic (b and e) or thymic (c and f) cells from mock-infected (a, b, and c) or MHV3-infected (d, e, and f) C57BL/6 mice. The cell preparations were labelled with FITC anti-CD44 mAb and analysed by flow cytometry. These results are representative of three different experiments.

Table 5. TCR-αβ expression levels in B220+ lymphocytes isolated from liver, and thymus from C57BL/6 mice at 72 hr after infection with MHV3

| Organ   | Virus    | TCRlow | TCRint | TCRhigh* |
|---------|----------|--------|--------|----------|
| Liver   | Uninfected | 29.34 ± 5.19 | 13.58 ± 3.54 | 1.73 ± 0.17 |
|         | MHV3     | 16.85 ± 4.18† | 13.36 ± 0.37 | 3.68 ± 0.20† |
| Thymus  | Uninfected | 0.05 ± 0.02  | 0.06 ± 0.08  | 0.41 ± 0.17  |
|         | MHV3     | 0.04 ± 0.02  | 0.10 ± 0.06  | 1.39 ± 0.21† |

*The cell preparations were double-labelled with FITC anti-B220 mAb or anti-CD44, and PE anti-αβ TCR mAb, and analysed by flow cytometry. Data are means ± SD of three mice per group. These results are representative of three different experiments.

†P<0.001.

and high cells in mock- and MHV3-infected mice (results not shown). In contrast, few hepatic B220+ cells, double-labelled with PI, were hypoploids (0-20 ± 0.12%) in mock-infected mice, but increased to 2.25 ± 1.42% in MHV3-infected C57BL/6 mice, whereas lower levels of hypoploids cells were detected in the thymus of mock-infected (0.04 ± 0.03%) or MHV3-infected (0.02 ± 0.02%) mice. Taken together, these results indicate that MHV3 infection does not induce a higher level of apoptotic αβ-TCR+ cells in the liver or thymus and does not support the hypothesis of accumulation of apoptotic cells in the liver during viral infection.

Viral replication in hepatic, splenic and thymic MNC from MHV3-infected C57BL/6 mice

Endothelial and/or Kupffer cells, involved in the stimulation of inflammatory responses and antigen-presenting activity, may serve as a reservoir of infectious viruses able to stimulate resident T-cell populations. Although thymic and splenic T cells are not reported as permissive to MHV3 replication,19 we have analysed viral replication in liver-resident MNC from MHV3 infected-C57BL/6 mice. Infectious viruses or viral proteins were detected, respectively, by viral titration onto L2 cells or immunofluorescence labelling with a polyclonal anti-MHV3 antiserum on cytocentrifuged-L2 cells. Viral titres found were comparable in hepatic MNC (105±2.0 TCID50/10⁶ cells) as in splenic cells (104±2.0 TCID50/10⁶ cells) or thymic cells (104±2.0 TCID50) and, probably represent residual viruses. In addition, no significant percentage of viral protein expressing cells was found in hepatic lymphocytes (less than 5%), suggesting that liver MNC are not the major target cells for MHV3 replication.

DISCUSSION

In the present study, we report the induction of an extrathymic differentiation process in the resident hepatic T-cell subpopulation during acute MHV3-induced hepatitis in susceptible C57BL/6 mice. Shifts of αβ-TCRint toward αβ-TCRhigh T cells were found in the liver and thymus from infected mice, but not in the spleen. The number of liver-resident MNC increased during the viral infection, in contrast to a decrease in thymic cellularity. DP cell populations, present in liver and thymic cell preparations from mock-infected C57BL/6 mice, decreased following viral infection. SP CD4+ cells, however, strongly increased in both the liver and thymus. The intrahepatic αβ-TCRint DN cell population also decreased following viral infection. The specific hepatic αβ-TCRint or high LFA-1high cell population, however, increased in the liver of infected mice. Expression profile patterns of CD44 (Pgp-1) and CD45R (B220) indicate that resident hepatic αβ-TCRhigh cells were activated or antigen experienced during viral infection.

MHV3 is the most pathogenic and hepatotropic strain of MHV viruses.8 It was previously reported that susceptible
MHV3 infection induces the extrathymic differentiation of liver-resident TCR<sup>ab</sup> T-cell population

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...mice, such as C57BL/6, died within 72 hr post-infection from a fulminant hepatitis. Histopathological study has reported extensive lesions, characterized by focal necrosis and the presence of inflammatory cells in MHV3 infected-C57BL/6 mice. Liver-resident MNC increased in MHV3-infected C57BL/6 mice at 72 hr p.i. We have found, in our hepatic cell preparations from mock-infected C57BL/6, the \( \text{z} \& \text{b}-\text{TCR} \) and \( \text{h} \& \text{g} \) SP CD4<sup>+</sup> or CD8<sup>+</sup>, and the DN \( \text{z} \& \text{b}-\text{TCR} \) or high cell populations, reported by Watanabe et al. Few DP cells, similar to those seen in the thymus and normally involved in positive or negative selection, occurred in our liver-resident MNC preparations. Major thymocyte development steps are defined by a progression from DN (CD4<sup>+</sup> CD8<sup>+</sup>) \( \text{z} \& \text{b}-\text{TCR} \) to DP (CD4<sup>+</sup> CD8<sup>+</sup>) \( \text{z} \& \text{b}-\text{TCR} \) immature cells, and finally to SP CD4<sup>+</sup> or CD8<sup>+</sup> effector cells expressing a \( \text{z} \& \text{b}-\text{TCR} \). Recently, an extrathymic differentiation pathway has been reported with \( \text{z} \& \text{b}-\text{TCR} \) and \( \gamma \& \delta-\text{TCR} \) T lymphocytes showing different properties from intrathymic T cells. Liver-resident T cells have an \( \text{z} \& \text{b}-\text{TCR} \) of intermediate expression \( \text{z} \& \text{b}-\text{TCR} \) and consist of DN and SP CD4<sup>+</sup> or CD8<sup>+</sup> in older mice. \( \text{z} \& \text{b}-\text{TCR} \) cells appear in systemic lymphoid organs, including the peripheral blood, bone marrow, and lymph nodes, and ultimately replace thymus-derived T cells. Thus, the thymus is crucial for forming the T-cell pool in early life, but thereafter the T-cell pool is self-sufficient. It was proposed that naive T cells leaving the thymus are destined to remain in interphase until encounter a specific antigen. It was shown, in athymic mice, that only \( \text{z} \& \text{b}-\text{TCR} \) cells were present in the liver and peripheral immune organs, indicating that immature thymocytes may emigrate from thymus toward other organs.

Viral infection could activate the hepatic extrathymic pathway since SP CD4<sup>+</sup> or CD8<sup>+</sup> cells expressing a \( \text{z} \& \text{b}-\text{TCR} \) became more abundant in the liver of MHV3-infected C57BL/6 mice. These cells could come from resident DN or SP CD4<sup>+</sup> or CD8<sup>+</sup> expressing an intermediate level of \( \text{z} \& \text{b}-\text{TCR} \) molecules. This hypothesis is supported by the fact that the DN \( \text{z} \& \text{b}-\text{TCR} \) or high, observed in the liver MNC from mock-infected animals, decreased during the viral infection. Presence of DN \( \text{z} \& \text{b}-\text{TCR} \) T cells has been reported in the liver of normal mice, occurring more frequently as the animals get older, whereas this cell population was also found in the liver of congenitally athymic mice. This raises the possibility that these cells are generated in the liver and do not constitute thymic emigrants.

It is of note that while the thymus was particularly depleted during MHV3 infection in C57BL/6, liver-resident MNC cellularity actually increased. Watanabe et al. have already noted that activation of the extrathymic T-cell differentiation pathway is always accompanied by thymic atrophy. We have previously reported that thymic atrophy is caused by the permissivity of thymic stromal cells to a lytic MHV3 infection subsequently leading to thymocyte lysis. The extrathymic differentiation pathway, as seen in the liver and intestinal epithelium, becomes predominant with ageing, concurrently with thymic involution, thereby suggesting that extrathymic pathways may be activated when the intrathymic pathway is inactivated. In addition, thymic atrophy accompanies the stimulation of the extrathymic differentiation pathway, as seen in malignancies, bacterial infections or autoimmune diseases. Since the thymic atrophy induced by MHV3 infection in C57BL/6 mice occurs in the first 48 hr of infection, our results suggest that the hepatic extrathymic differentiation pathway would then be activated. We have observed a decrease in absolute numbers of all thymocyte subpopulations while the percentage of thymic SP CD4<sup>+</sup> \( \text{z} \& \text{b}-\text{TCR} \) cells increased during viral infection. This observation may be explained by a faster up-regulation of TCR density in SP CD4<sup>+</sup> cell production than in SP CD8<sup>+</sup> cell generation. Indeed, these authors demonstrated a lag between TCR<sup>ab</sup> and SP cell generation, as the high TCR expression occurred first in immature DP cells, and the maturation transition from DP to SP is restricted to TCR<sup>ab</sup> cells. In addition, SP CD4<sup>+</sup> cells are known to appear earlier than SP CD8<sup>+</sup> cells, as their precursor remains at the double-positive stage two days longer than thymic SP CD4<sup>+</sup> cells. The increase of TCR<sup>ab</sup> SP CD4<sup>+</sup> cells in MHV3-infected mice suggests that the signal for SP cells generated by DP precursors may be different from that up-regulating \( \text{z} \& \text{b}-\text{TCR} \). On the other hand, thymic \( \text{h} \& \text{g}-\text{TCR} \) SP CD4<sup>+</sup> could be mature splenic cells mounting an immune response against viral antigens from infected stromal cells. This hypothesis is not supported by a splenic T-cell stimulation, since no increase in the number of splenic lymphoid cells or in the percentage of SP CD4<sup>+</sup> cells was evidenced in MHV3-infected mice. In addition, hepatic TCR<sup>ab</sup> or high cells express a high level of LFA-1 molecules. The LFA-1/ICAM-1 interaction plays an important role not only in the pathogenesis of inflammation but also in T-cell differentiation in the thymus, suggesting that this interaction contributes to T-cell differentiation in the liver. However, mechanisms involved in the reciprocal interaction between the T-cell differentiation pathways in the thymus and liver are not yet elucidated.

The fact that the thymus was rapidly depleted in cells showing immature phenotypes in MHV3-infected mice does not support thymic cell emigration toward the liver. The increase of TCR<sup>ab</sup> SP T cells in the liver from MHV3-infected mice, but not in the spleen, suggests that the differentiation process may occur in situ, during the first days of infection, in response to MHV3-mediated cellular injuries in the liver. The MHV3 serotype possesses a tropism for several hepatic cells, such as hepatocytes, Kupffer, Ito and endothelial cells in susceptible mice. Viral RNA and antigens appear during the first 24 hr post-infection in the liver, and are first detectable in Kupffer<sup>0</sup> and endothelial cells. Martin et al. postulated that after viral multiplication and lysis of these cells, the virus reaches the parenchymal cells, where its multiplication leads to focal necrosis. The pathogenic processes involved in the hepatitis are not clearly established but are not related directly to viral replication, as comparable virus titres were found in susceptible C57BL/6, resistant A/J, mice, as well as in susceptible mice infected with the non-pathogenic YAC-MHV3 variant, despite major differences in the histopathological lesions. On other hand, induction of procoagulant activity (PCA) correlated with resistance/susceptibility to infection in different mouse strains. Both macrophages and endothelial cells expressed detectable PCA suggesting that PCA may exert its effect through activation of the coagulation system leading to vascular thrombosis. Some pathological observations suggest that the hepatocytes at inflammation sites express ICAM-1 antigen and that thrombin can also induce ICAM-1 expression in hepatic endothelial cells. We have observed
that MHV3 infection favours the expression of the CD44 marker by hepatic lymphocytes. Thus, the high level of CD44 expression in hepatic TCR\(^{\text{int}}\) or \(\text{high}\) T cells from MHV3-infected mice indicates the ability of such hepatic lymphoid cells, possibly activated, to interact with endothelial cells.\(^{33,44}\) Pope \textit{et al.}\(^{45}\) reported the existence of a splenic T-helper 2 (Th2: CD4\(^+\)) subpopulation more effective in stimulating macrophage PCA following MHV3 infection in susceptible mice. In addition, induction of PCA by MHV3 requires live virus as well as host protein and RNA synthesis, suggesting that viral replication in some target cells is an essential event for the activation of the intravascular coagulation system.\(^{46}\) Stromal thymic or hepatic endothelial cells or macrophages may therefore act as target cells to viral infection and, at the same time, act as antigen-presenting cells able to activate liver-resident T cells. Hepatic CD44\(^{\text{high}}\) T cells may thus be involved in the PCA activation.

Our results suggest that extrathymic differentiation of resident hepatic cells may be an early event resulting probably from viral infection of non-lymphoid target cells in the liver and thymus. The rapid intravascular coagulation induced by PCA may block the recruitment of peripheral activated T cells, favouring the activation of liver resident T cells. Huang \textit{et al.}\(^{33}\) have reported that, as early as 12 hr after antigenic peptide injection, hepatic T cells undergo blastogenesis followed by the occurrence of B220 and CD25 markers, but that a down-regulation of all other surface markers occurs after four days. We did not find any evidence of a significant occurrence of B220\(^+\) T cells in the liver from MHV3-infected mice, nor of a down-regulation of other accessory molecules until the death of the animals at three days p.i. We did not observe any increase among splenic mature T-cell populations in MHV3-infected mice, as would be expected in an antigen-driven immune cell activation, reinforcing the hypothesis of a resident hepatic T cell in \textit{situ} differentiation.

We have previously shown that MHV3 infection induces a cellular immunodeficiency in susceptible C57BL/6 mice, as early as 48 hr p.i.\(^{12}\) In addition, MHV viruses are known to suppress the modulation of splenic T-cell activation, even when T-cell subset proportions are not altered.\(^{47}\) We can thus speculate that the extrathymic T-cell differentiation pathway stimulation may be a mechanism induced to protect the liver in the first days of MHV3 infection, although the precise protective role of hepatic \(\alpha\beta\text{-TCR}\text{int}\) or \(\text{high}\) T cells against viral infection is not yet known. However, it has been reported that hepatic \(\alpha\beta\text{-TCR}\text{int}\) LFA-1\(^{\text{high}}\) T cells protected mice in early stages of salmonella infection while \(\alpha\beta\text{-TCR}\text{high}\) LFA-1\(^{\text{high}}\) cells acted on the later stages of the infection.\(^{27}\) Preliminary results using a non-pathogenic YAC-MHV3 variant suggest that these cell populations may induce a protective effect against hepatitis. In addition, the high LFA-1 expression on these cells indicates that they can bind to endothelial cells and act as a potent stimulatory signal for antigen-specific T-cell proliferation, thereby enhancing interleukin-2 production.\(^{48}\)

At this time, we have no information yet on the ability of specific hepatic T-cell subpopulations to mount an efficient local immune response against MHV3-infected target cells. Work is in progress to analyse the ability of \(\alpha\beta\text{-TCR}\text{int}\) and \(\text{high}\) to control viral replication in hepatic target cells.

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