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

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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^{intermediate} T cells toward $\alpha\beta$-TCR^{high} was evidenced in the liver and thymus of infected mice, but not in the spleen. The few $\alpha\beta$-TCR^{int} double negative (DN) ($CD4^-CD8^-$) cells also decreased following viral infection. $\alpha\beta$-TCR$^{int\ or\ high}$ 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.[1–3] 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.[4] 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.[5,6] 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.[5,6]

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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.[7–9] 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.[10] (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.[11] 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.[12]

Several factors are involved in the pathogenic process of hepatitis. Viral replication in various hepatic cells, such as Kuppfer cells, endothelial cells, and hepatocytes,[13–15] 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^{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^{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 oligoclones 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^{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 in spleen, the number of hepatic MNC actually increased during the viral infection. Hepatic $\alpha\beta$-TCR$^{low\ or\ int}$ shifted to $\alpha\beta$-TCR$^{int\ or\ high}$ T cells in infected mice. In addition, the specific hepatic $\alpha\beta$-TCR^{int}, LFA-1^{high} cell population increased in the liver of MHV3-infected mice and remained high in hepatic and thymic $\alpha\beta$-TCR$^{int\ or\ high}$ T cells. Studies with activation markers suggest that hepatic $\alpha\beta$-TCR^{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-μm 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 μm cell strainer, which was then washed with 20 ml of RPMI-1640 (Gibco Laboratories, Grand Island, NY) contained 20% SVF 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

Cytofluorometry. Double immunolabelling was performed as follows: 10^5 cells were resuspended in 500 μl of RPMI-1640 containing 20% FCS, and incubated on ice for 1 hr with the optimal dilution of monoclonal antibody (mAb) anti-mouse CD4–fluoroscein 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 αβ-TCR–PE: clone H57-597, Pharmingen), LFA-1/αβ-TCR (mAb anti-mouse CD11a–FITC: clone M17/4, Pharmingen), $CD4^+$ CD8/αβ-TCR, CD11b/αβ-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)/αβ-TCR, and μ chains (polyclonal goat anti-mouse μ 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.*[29] Cells labelled with FITC-conjugated anti-αβ-TCR, anti-B220 or anti-CD44 antibodies were fixed overnight in 0·1% formaldehyde at 4°, 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/mg and 50 μg/ml PI) at 4°.

Cytofluorometric 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 subpopulations. 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 4×10^5 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 (immunoglobulin G: IgG) (Dimension Laboratories, Missisauga, Ontario, Canada), incubated for an additional 30 min, and rewashed in PBS. Double immunolabelling using mAb anti-Mac-1–FITC (clone M1/70: Pharmingen), in conjunction with polyclonal anti-MHV3 antiserum 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°, 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 $\log_{10}$ 50% $TCID_{50}$. 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–Witney *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 marow 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 $TCID_{50}$ of MHV3 virus (10 $TCID_{50}$ is equivalent to one LD_{50}). 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 ($P < 0{\cdot}001$). 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-resident MNC suspensions from mock-infected C57BL/6 mice revealed that MNC represent more than 90% of the cells. Few macrophages (less than 3%) ($CD11b^+$) or 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

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

	Cellularity (10^6)		
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.

†$P < 0{\cdot}001$.

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.[24] 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. Morever, 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-TCR^{int} cells, normally only present in thymus, were also found among the liver-resident MNC suggesting an extrathymic differentiation pathway.[23,24] αβ-TCR^{int} cells from the liver, but not from the thymus, may act against bacterial infections.[27] To verify the role of MHV3 infection in the *in situ* differentiation of hepatic T cells, we analysed the αβ-TCR profile patterns of lymphocytes from the liver, spleen and thymus of mock-infected and MHV3-infected mice. αβ-TCR$^+$ cells were further separated in TCR^{int} and TCR^{high} based on the immunofluorescence profiles of spleen cells, as previously described.[23] Single-colour αβ-TCR analysis of hepatic, splenic and thymic MNC showed

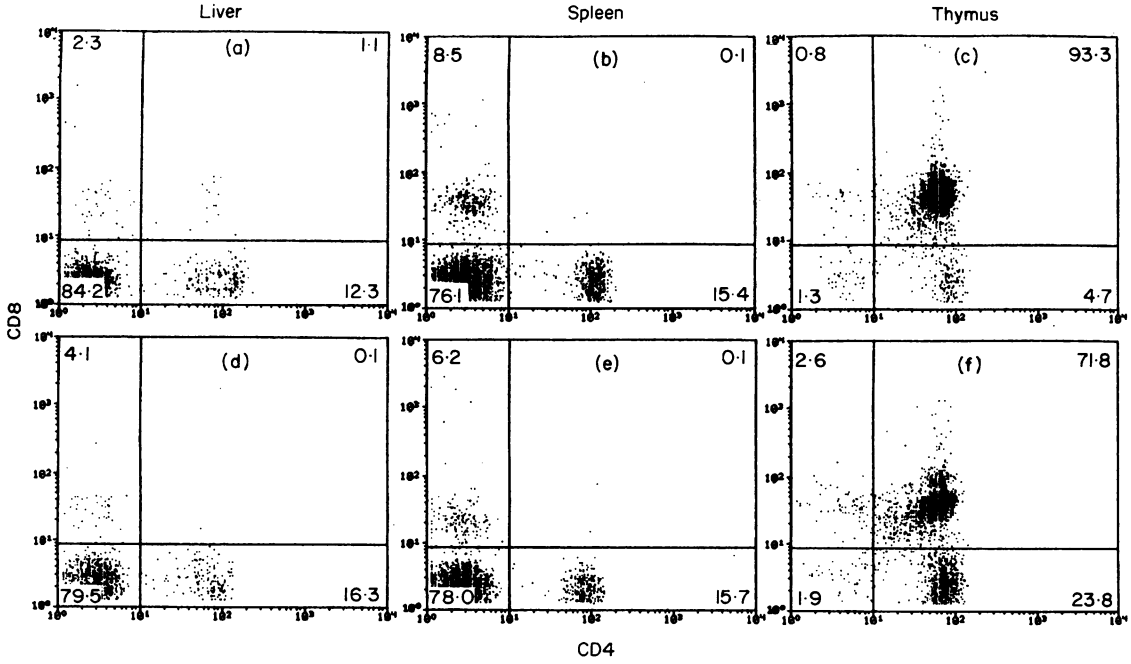

Figure 1. Phenotypic characterization with CD4, and CD8 antigens of hepatic MNC (a and d), splenic (b and e) or thymic (c and f) cells from mock-infected (a,b, and c) or MHV3-infected (d,e, and f) C57BL/6 mice. The cell preparations were double-labelled with FITC anti-CD4 mAb, and PE anti-CD8 mAb, and analysed by flow cytometry. Numbers indicate the percentage of fluorescence-positive cells in the corresponding squares. These results are representative of three different experiments.

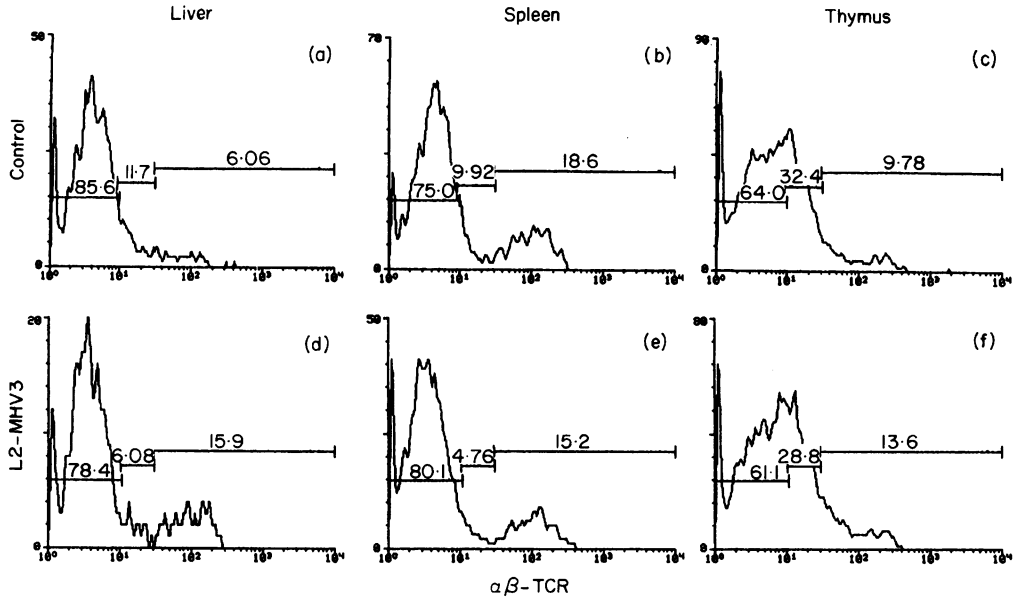

Figure 2. A comparison of expression profiles (single-colour staining of αβ-TCR) of hepatic MNC (a and d), splenic (b and e) or thymic (c and f) cells from mock-infected (a,b, and c) or MHV3-infected (d,e, and f) C57BL/6 mice. The cell preparations were labelled with PE anti-ab-TCR mAb and analysed by flow cytometry. Numbers indicate the percentage of αβTCR$^{low,int,or\ high}$ T-cell subpopulations. These results are representative of three different experiments.

a shift toward αβ-TCR^{high} T cells in hepatic and thymic MNC from MHV3 infected C57BL/6 mice, as evidenced by decreases in αβ-TCR^{low} and αβ-TCR^{int} cells, and by an increase in αβ-TCR^{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 αβ-TCR revealed that percentages of hepatic and thymic αβ-TCR^{high} CD4$^+$ cells in total lymphoid cells increased strongly in MHV3-infected C57BL/6 mice ($P<0{\cdot}001$), whereas thymic αβ-TCR^{low} CD4$^+$ cells decreased ($P<0{\cdot}001$: Table 3). All CD4 cell subpopulations decreased in the spleen of infected C57BL/6 mice ($P<0{\cdot}01$ to $P<0{\cdot}001$). Comparable results were also observed with hepatic, splenic and thymic SP CD8$^+$ cells (results not shown). The increase of αβ-TCR^{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 αβ-TCR^{high} CD4$^+$ cells in MHV3-infected C57BL/6 mice can reflect the higher loss of αβ-TCR^{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.

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

Organ	Virus	TCR^{low}	TCR^{int}	TCR^{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-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.

†$P<0{\cdot}001$.

‡$P<0{\cdot}01$.

A specific liver-resident DN αβ-TCR^{int} cell population has also been reported.[22,23] If the viral infection stimulates an intrahepatic immature T-cell differentiation pathway, αβ-TCR$^{int\ or\ high}$ DN cell levels should decrease. To test this hypothesis, we analysed the αβ-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 αβ-TCR$^{int\ or\ high}$ DN cells, as well as an increase of SP CD4$^+$ and/or CD8$^+$ cells. In the thymus, few αβ-TCR$^+$ DN cells were also detected. Percentages of αβ-TCR$^{int\ or\ high}$ DN thymic cells increased slightly in MHV3-infected C57BL/6 mice (αβ-TCR:^{int} 0·02±0·01–0·19±0·06; αβ-TCR:^{high} 0·07±0·02–0·21±0·05) ($P<0{\cdot}001$), reflecting the important decrease in cells in the atrophic thymus rather than an increase in the number of such DN cells.

αβ-TCR–LFA-1 profile patterns of hepatic, splenic and thymic T lymphocytes isolated from C57BL/6 mice infected with MHV3

Adhesion of T cells to the vascular endothelium, antigen-presenting cells or target cells in a cytotoxic reaction depends on adhesion molecules, such as LFA-1.[30,31] Matsumoto *et al.* have recently demonstrated the protective role of specific hepatic αβ-TCR^{int} T cells, expressing a high intensity of LFA-1, in the resolution of murine salmonellosis.[32] intercellular adhesion molecule-1 (ICAM-1)/LFA-1 interaction can also be

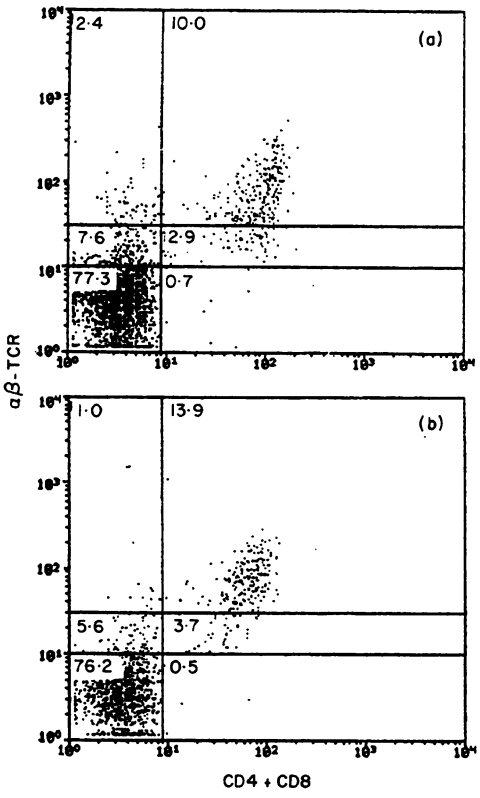

Figure 3. Phenotypic characterization with CD4 and CD8, and $\alpha\beta$-TCR antigens of hepatic MNC from mock-infected (a) or L2-MHV3-infected (b) C57BL/6 mice. The cell preparations were double-labelled with FITC anti-CD4 mixed with FITC anti-CD8 mAbs, and PE anti-$\alpha\beta$-TCR mAb, and analysed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

a potent costimulatory signal for antigen-specific T-cell proliferation.[31] To determine if $\alpha\beta$-TCR$^{int\ or\ high}$ T cells, induced in liver during MHV3 infection, express a high level of LFA-1 or therefore are stimulated to proliferate, C57BL/6 mice were infected with MHV3. The liver, spleen and thymus were collected at 72 hr p.i., and MNC from these organs were double-labelled for the LFA-1 and $\alpha\beta$-TCR markers. As shown in Table 4, in mock-infected mice, the percentage of $\alpha\beta$-TCR^{int} LFA-1^{high} cell population was higher among hepatic MNC than in splenic cells. The $\alpha\beta$-TCR$^{int\ or\ high}$ LFA-1^{high} cell populations strongly increased after MHV3 infection in C57BL/6 mice ($P<0{\cdot}001$). A shift toward $\alpha\beta$-TCR$^{int\ or\ high}$ LFA-1^{high} T cells was also seen in thymic cells from MHV3-infected mice ($P<0{\cdot}001$: Table 4). These results suggest that MHV3 infection induces an increase of $\alpha\beta$-TCR$^{int\ or\ high}$ LFA-1^{high} T cells in the liver, and to a lesser extent, in the thymus.

Table 4. TCR-$\alpha\beta$ expression levels in LFA-1^{high} lymphocytes isolated from liver, spleen and thymus from C57BL/6 mice at 72 hr after infection with MHV3

Organ	Virus	TCR^{low}	TCR^{int}	TCR^{high}*
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	2·3±0·9	10·4±3·5
	MHV3	42·4±18·8	4·4±2·7	17·0±8·7
Thymus	Uninfected	73·7±9·5	23·4±9·6	2·4±0·3
	MHV3	40·7±13·5†	43·5±6·8†	16·4±3·9†

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

†$P<0{\cdot}001$.

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$^+$ have to be activated. It was previously reported that many hepatic $\alpha\beta$-TCR^{int} express IL-2Rβ and CD44 (Pgp-1) cell surface markers, as seen on antigen-experienced T cells,[21–22] 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 seen in Fig. 4(a), higher number of CD44^{high} cells was found in hepatic MNC than in spleen or thymus from mock-infected mice (Fig. 4b,c). CD44^{high} 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 TCR^{int}CD44^{high} cell population in liver increased strongly in MHV3-infected mice (12·89±3·52–20·30±2·25%) ($P<0{\cdot}01$), 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.[33] Such activated cells express the CD45R (B220) marker and accumulate in the liver where they undergo apoptosis.[33,34] To verify if increase in hepatic MNC results from the accumulation of such cells, percentages of B220$^+$ cells among $\alpha\beta$-TCR$^{int\ or\ high}$ cells in the liver and thymus were analysed in mock-infected or MHV3-infected mice. As shown in Table 5, percentages of B220$^+$ $\alpha\beta$-TCR^{high} 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, splenic and thymic MNC preparations in mock-infected or MHV3-infected mice. As expected, we found a higher percentage of hypoploid cells in the liver MNC preparations (4·51±0·95%) than in splenic cells (0·12±0·05%) or thymocytes (0·05±0·01%) from mock-infected mice. In contrast, hypoploid hepatic MNC decreased in MHV3-infected mice (1·67±1·35%). To verify if the hepatic $\alpha\beta$-TCR$^+$ cells were hypoploids, we double-labelled these cells with PI and mAb FITC-conjugated anti-$\alpha\beta$-TCR. Similar percentages of hypoploid cells were found in $\alpha\beta$-TCR^{int}

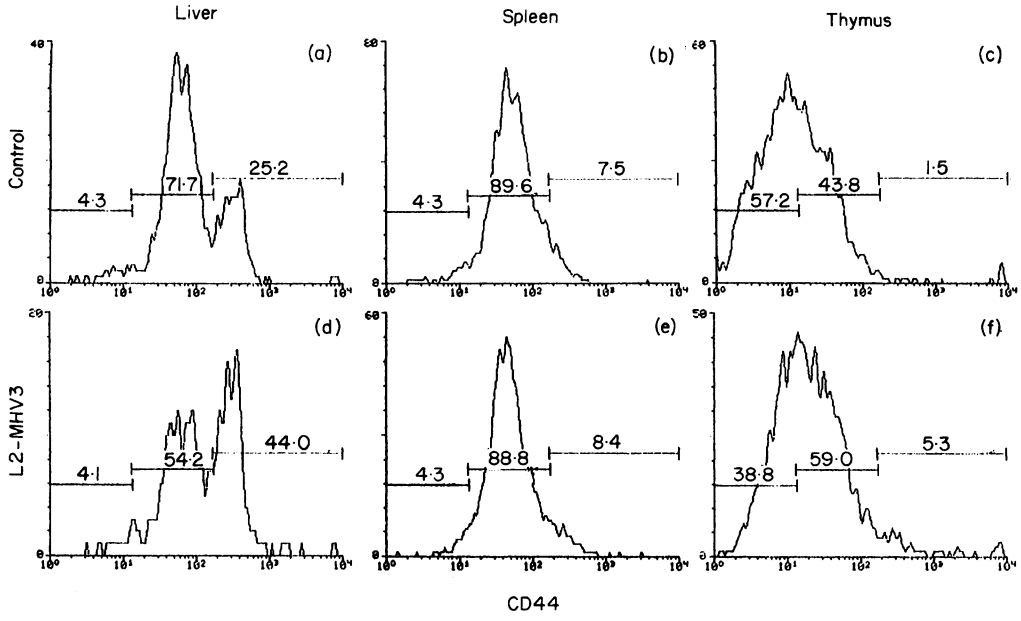

Figure 4. A comparison of expression profiles (single-colour staining of CD44) of hepatic MNC (a and d), splenic (b and e) or thymic (c and f) cells from mock-infected (a,b, and c) or MHV3-infected (d,e, and f) C57BL/6 mice. The cell preparations were labelled with FITC anti-CD44 mAb and analysed by flow cytometry. These results are representative of three different experiments.

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

Organ	Virus	TCR^{low}	TCR^{int}	TCR^{high}*
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-$\alpha\beta$ TCR mAb, and analysed by flow cytometry. Data are means ± SD of three mice per group. These results are representative of three different experiments.

†$P < 0{\cdot}001$.

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 hypoploid 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 $\alpha\beta$-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 Kuppfer 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 ($10^{2{\cdot}7 \pm 0{\cdot}7}$ $TCID_{50}/10^6$ cells) as in splenic cells ($10^{4{\cdot}2 \pm 0{\cdot}3}$ $TCID_{50}$) or thymic cells ($10^{4{\cdot}2 \pm 0{\cdot}2}$ $TCID_{50}$) 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 $\alpha\beta$-TCR^{int} toward $\alpha\beta$-TCR^{high} 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 $\alpha\beta$-TCR^{int} DN cell population also decreased following viral infection. The specific hepatic $\alpha\beta$-$TCR^{int\ or\ high}$ $LFA\text{-}1^{high}$ cell population, however, increased in the liver of infected mice. Expression profile patterns of CD44 (Pgp-1) and CD45R (B220) indicate that resident hepatic $\alpha\beta$-TCR^{high} 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

mice, such as C57BL/6, died within 72 hr post-infection from a fulminant hepatitis.[9] Histopathological study has reported extensive lesions, characterized by focal necrosis and the presence of inflammatory cells in MHV3 infected-C57BL/6 mice.[12] 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 $\alpha\beta$-TCR$^{int\ and\ high}$ SP CD4$^+$ or CD8$^+$, and the DN $\alpha\beta$-TCR$^{int\ or\ high}$ cell populations, reported by Watanabe *et al.*[23] Few DP cells, similar to those seen in the thymus and normally involved in positive or negative selection,[35] occurred in our liver-resident MNC preparations. Major thymocyte development steps are defined by a progression from DN (CD4$^-$ CD8$^-$) $\alpha\beta$-TCR^{low} to DP (CD4$^+$ CD8$^+$) $\alpha\beta$-TCR^{int} immature cells, and finally to SP CD4$^+$ or CD8$^+$ effector cells expressing a $\alpha\beta$-TCR^{high}.[35] Recently, an extrathymic differentiation pathway has been reported with $\alpha\beta$-TCR$^+$ and $\gamma\delta$-TCR$^+$ T lymphocytes showing different properties from intrathymic T cells.[21–23] Liver-resident T cells have an $\alpha\beta$-TCR of intermediate expression ($\alpha\beta$-TCR^{int}) and consist of DN and SP CD4$^+$ or CD8$^+$.[22,23] In older mice, $\alpha\beta$-TCR^{int} cells appear in systemic lymphoid organs, including the peripheral blood, bone marrow, and lymph nodes, and ultimately replace thymus-derived T cells.[22] Thus, the thymus is crucial for forming the T-cell pool in early life but, thereafter the T-cell pool is self-sufficient.[36] 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 $\alpha\beta$-TCR^{int} cells were present in the liver and peripheral immune organs, indicating that immature thymocytes may emigrate from thymus toward other organs.[24]

Viral infection could activate the hepatic extrathymic pathway since SP CD4$^+$ or CD8$^+$ cells expressing an $\alpha\beta$-TCR^{high} became more abundant in the liver of MHV3-infected C57BL/6 mice. These cells could come from resident DN or SP CD4$^+$ and CD8$^+$ cells expressing an intermediate level of $\alpha\beta$-TCR molecules. This hypothesis is supported by the fact that the DN $\alpha\beta$-TCR$^{int\ or\ high}$, observed in the liver MNC from mock-infected animals, decreased during the viral infection. Presence of DN $\alpha\beta$-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 congenically athymic mice.[22] 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.[23] 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.[20] 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.[26] In addition, thymic atrophy accompanies the stimulation of the extrathymic differentiation pathway, as seen in malignancies, bacterial infections or autoimmune diseases.[23–26] 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$^+$ $\alpha\beta$-TCR^{high} cells increased during viral infection. This observation may be explained by a faster up-regulation of TCR density in SP CD4$^+$ cell production than in SP CD8$^+$ cell generation.[37] Indeed, these authors demonstrated a lag between TCR^{high} expressions and SP cell generation, as the high TCR expression occurred first in immature DP cells, and the maturational transition from DP to SP is restricted to TCR^{high} cells. In addition, SP CD4$^+$ cells are known to appear earlier than SP CD8$^+$ cells, as their precursor remains at the double-positive stage two days longer than future SD CD4$^+$ cells. The increase of TCR^{high} SP CD4$^+$ cells in MHV3-infected mice suggests that the signal for SP cells generated from DP precursors may be different from that up-regulating $\alpha\beta$-TCR expression.[38] On the other hand, thymic TCR^{high} SP CD4$^+$ 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$^+$ cells was evidenced in MHV3-infected mice. In addition, hepatic TCR$^{int\ or\ high}$ 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,[39] 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^{high} 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.[14] Viral RNA and antigens appear during the first 24 hr post-infection in the liver, and are first detectable in Kupffer[40] and endothelial cells.[14] Martin *et al.*[15] 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.[11,20] On other hand, induction of procoagulant activity (PCA) correlated with resistance/susceptibility to infection in different mouse strains.[16] 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[41] and that thrombin can also induce ICAM-1 expression in hepatic endothelial cells.[42] 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$^{int\ or\ high}$ T cells from MHV3-infected mice indicates the ability of such hepatic lymphoid cells, possibly activated, to interact with endothelial cells.[43,44] Pope *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^{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 *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 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 $\alpha\beta$-TCR$^{int\ or\ high}$ T cells against viral infection is not yet known. However, it has been reported that hepatic $\alpha\beta$-TCR^{int} LFA-1^{high} T cells protected mice in early stages of salmonella infection while $\alpha\beta$-TCR^{high} LFA-1^{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 $\alpha\beta$-TCR$^{int\ and\ high}$ to control viral replication in hepatic target cells.

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