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Recovery from Mouse Hepatitis Virus Infection Depends on Recruitment of CD8$^+$ Cells Rather Than Activation of Intrahepatic CD4$^+\alpha\beta^-$TCR^{inter} or NK-T Cells

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Mouse hepatitis virus (MHV) provides an excellent animal model for the study of the immunopathological mechanisms involved in hepatic viral diseases. We previously generated an attenuated viral variant, YAC-MHV3, which induces a subclinical disease and recovery within 15 days. In contrast, the L2-MHV3 strain induces the development of a fulminant hepatitis, leading to death within 3 days. In this paper, we document intrahepatic and splenic T cell subpopulations involved in the hepatitis process and viral elimination identified in attenuated or pathogenic MHV3-infected C57BL/6 mice. Percentages of intrahepatic CD4$^+$ cells decreased in attenuated YAC-MHV3-infected mice, while they increased in mice infected with pathogenic L2-MHV3, compared with uninfected animals. Moreover, in YAC-MHV3-infected mice, the percentages of intrahepatic CD8$^+$ cells slightly decreased at 24 h pi, then increased until 15 days pi. In contrast, the CD4/CD8 ratios of splenic lymphoid subpopulations increased in the first days of infection and returned to normal values at 15 days pi. Intrahepatic NK1.1$^+\alpha\beta$ − TCR^{inter} cells decreased in both virally infected groups of mice, while CD4$^+\alpha\beta$ − TCR^{inter} LFA-1^{high} cells increased in L2-MHV3-infected mice, in contrast with what was seen in YAC-MHV3-infected mice. However, these cells became anergic following Con A or PHA stimulation. *Ex vivo* studies showed that only the intrahepatic CD8$^+$ cells that were increased in YAC-MHV3-infected mice could be stimulated by lectins. In addition, *in vitro* viral infections revealed that L2-MHV3 viral infection led to an increase of intrahepatic CD4$^+\alpha\beta$ − TCR^{inter} cells in the absence of CD8$^+$ cells only. These results indicate that the attenuated phenotype of the YAC-MHV3 virus is related to two different mechanisms: the first involves no increase of intrahepatic CD4$^+\alpha\beta$ − TCR^{inter} or NK-T cells, while the second favors the recruitment and activation of CD8$^+$ cells in liver. The results are discussed in relation to the integrity of intrahepatic immune tolerance mechanisms and immune-mediated viral elimination.

Key Words: **MHV3; coronavirus; hepatitis; T lymphocytes; liver; suppression.**

INTRODUCTION

Hepatitis is an inflammatory disease induced by various causes, one of which is viral infection, in which T lymphocytes have been assumed to play an essential role in the host's immune response to tissue injury and elimination of viral infection (1–3). The incapacity of the host to eliminate the viral infection favors the development of acute or chronic hepatitis, such as that seen in human viral hepatitides B and C (4, 5). However, the roles of specific intrahepatic lymphoid subsets involved simultaneously in the immune responses against viral infection and in the intrahepatic immune tolerance may disturb the efficiency of immune-mediated viral elimination mechanisms in decreasing the intensity of the T-cell-mediated immune pathway (5).

Mouse hepatitis virus (MHV) infection provides an excellent animal model for the study of immune-mediated mechanisms involved in intrahepatic viral elimination. MHV3 is the most hepatotropic serotype, and the liver is the main target organ for viral replication in the susceptible C57BL/6 mouse (6, 7). The pathogenic L2-MHV3-infected mice develop an acute hepatitis and die within 3 days. Within this time, progressive necrotic lesions can be observed in the liver, inducing dysfunction due to extensive necrosis (7). Simultaneously, immune disorders occur in various lymphoid organs, leading to cellular and humoral immunodeficiences (7–10). We have previously generated a viral variant, YAC-MHV3, which induces a subclinical infection involving few hepatic cellular lesions in C57BL/6 mice, with viral clearance occurring within 15 days postinfection (pi) (8, 11). YAC-MHV3 does not induce any immunodeficiency, such as those observed with pathogenic L2-MHV3 in C57BL/6 infected mice (8–10). However, mechanisms involved in the attenuated liver pathogenicity of this variant have not yet been elucidated.

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The hepatic sinusoids of normal adult mice contain $\alpha\beta$ − TCR (T cell receptor) cells which appear to be distinct from the T cells found in peripheral lymphoid tissues (12–14). These T cells are characterized by an $\alpha\beta$ − TCR of intermediate intensity ($\alpha\beta$ − TCR^{inter}) and consist of double negative $CD4^-CD8^-$ and single positive $CD4^+$ or $CD8^+$ cells (13, 14). The percentage of single-positive $CD4^+$ cells is reported to be higher in the liver than in the spleen or thymus (14, 15). Moreover, these lymphocytes express a higher level of leucocyte function antigen-1 (LFA-1) (14), while some of them express the IL-2Rβ and PgP-1 (CD44) cell surface markers normally found on antigen-experienced T cells (12, 13, 16). Some other $\alpha\beta$ − TCR^{inter} cell subsets express natural killer (NK)1.1 molecules and are defined as NK-T cells (17). The protective role of these intrahepatic T cell subsets is contradictory. It has been demonstrated that intrahepatic $\alpha\beta$ − TCR^{inter} cells account for a protective mechanism in hepatic resistance to the early phase of murine salmonellosis (18). In contrast, down-regulation of $CD4^+\alpha\beta$ − TCR^{inter} cells favors the development of a protective immunity against listeriosis, and bacterial clearance has thus been mediated by cytotoxic $CD8^+$ cells (19, 20). No information is available on the protective role of these cells in viral hepatitis.

We have previously observed that infection of susceptible C57BL/6 mice with the pathogenic L2-MHV3 strain induced an increase in the total number of intrahepatic mononuclear cells (MNC) and in the CD4/CD8 ratio during acute hepatitis (21). Moreover, the hepatic $\alpha\beta$ − $TCR^{inter}LFA\text{-}1^{high}$ and $\alpha\beta$ − TCR^{high} $LFA\text{-}1^{high}$ cells increased. Studies with activation markers suggest that hepatic $\alpha\beta$ − $TCR^{inter}LFA\text{-}1^{high}$ cells may be activated *in situ* during viral hepatitis but are not able to control the viral infection, suggesting that they are not protective against viral infection. Thus, the use of an attenuated virus that induces a subclinical infection without extensive hepatic lesions may be useful in elucidating the phenotype of the protective intrahepatic T cell subpopulations.

In this paper, we report on the hepatic and splenic T cell subpopulations during viral infection induced by the attenuated YAC-MHV3 variant in susceptible C57BL/6 mice. The results indicated that the attenuated phenotype of the YAC-MHV3 virus was related to two different mechanisms: the first involved the absence of intrahepatic $CD4^+\alpha\beta$ − TCR^{inter} or NK-T cell activation and the second favored the recruitment and activation of $CD8^+$ cells in liver.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Prior to experimentation, the animals were tested for the presence of anti-MHV antibodies by an ELISA test using a MHV3 preparation as antigen. During experimentation, the animals were housed in a sterile atmosphere (Forma Scientific, Marietta, OH). Female mice between 8 and 12 weeks of age were used in all experiments.

Virus

Pathogenic MHV3 is a cloned substrain isolated from the liver of infected DBA2 mice and passaged two or four times into L2 cells (L2-MHV3). Attenuated MHV3 is a cloned substrain produced in YAC-1 cells as previously described (YAC-MHV3) (11). Viruses were passaged in L2 cells before use and their pathogenic properties were tested regularly.

In vivo viral infections. Groups of three mice were infected intraperitoneally (ip) with 1000 $TCID_{50}$ of pathogenic L2-MHV3 or the attenuated YAC-MHV3 variant (8). Mock-infected mice received ip a similar volume of PBS. At various times pi, mice were anesthetized with ketamine sulphate (200 mg/kg) and xylazine (10 mg/kg) by ip injection. Mice were bled by section of portal veins and aortic arteries, as described by Watanabe *et al.* (14). Liver and spleen were harvested following exsanguination.

Cells

L2 cells, a continuous mouse fibroblast cell line, were grown in Dulbecco's MEM with glutamine (2 mM), 5% fetal calf serum (FCS), and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 mg/ml) (GIBCO BRL, Grand Island, NY). L2 cells were used for virus production and titrations.

Splenic lymphocytes were obtained from three mice in each experimental group. Spleens were pressed through a 70-μm cell strainer (Falcon, Fisher Scientific Co., Montréal, Québec, Canada) into cold RPMI 1640 supplemented with 20% FCS (GIBCO). Lymphocytes were enriched by passage through a Lymphoprep gradient (Cedarlane, Hornby, Ontario, Canada). The cell suspensions were then centrifuged, resuspended in 1 ml of RPMI 1640 with 20% FCS, and electronically counted (Coulter Counter, Coulter Electronics, Hialeah, FL); cell viability, ranging from 90 to 100%, was assayed by trypan blue exclusion.

Livers from three mice in each experimental group were pressed through a 70-μm cell strainer to obtain MNC. The cells were then washed with 20 ml of RPMI 1640 (GIBCO) containing 20% FCS and antibiotics. The cell suspensions were pooled and 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 and 67% Percoll in PBS) (Am-

ersham Pharmacia, Uppsala, Sweden) for 30 min at 1000*g*. 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.

Depletion of CD8$^+$ cells in hepatic and splenic cells was done by incubation of cells with anti-CD8 antibodies (monoclonal antibody (mAb) 53-6.7, Pharmingen, San Diego, CA) and guinea pig complement (Cedarlane). Residual cells were phenotyped 24 h later, as described below.

In vitro Viral Infections

MNC were isolated from livers originating from groups of 6 to 10 mice, as described above, and pooled together. Cells were seeded in 24-well plates at a concentration of 10^6 cells/ml and infected with 0.01 to 1 m.o.i. of L2-MHV3 or YAC-MHV3 viruses. The plates were incubated at 37°C under a CO_2 atmosphere for 24 h, and cells in the supernatants were collected, electronically counted, and immunolabeled for cytofluorometric studies.

Virus Titration

Livers from infected mice were collected in RPMI 1640, 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 microtiter plates. Cytopathic effects, characterized by syncytia formation and cell lysis, were recorded at 72 h pi and virus titers expressed as $\log_{10}$ 50% $TCID_{50}$. All titrations were performed in triplicate.

Double Immunolabelings

T cell subpopulations were labeled with the following mAb: fluorescein isothiocyanate-conjugated (FITC)-anti-CD4 (mAb RM45, Pharmingen), phycoerythrine-conjugated (PEC)-anti-CD8a (mAb 53-6.7, Pharmingen), PEC-NK1.1 (clone PK-136, Pharmingen), FITC- or PEC-anti-TCR (clone H57-597, Pharmingen), FITC-anti-LFA-1 (clone M17/4, Pharmingen), FITC-anti-B220 (clone RA3-6B2, Pharmingen) and PEC-anti-CD44 (clone IM7, Pharmingen). Cell suspensions were prepared from the spleen, and 10^6 cells were incubated with an optimal concentration of mAb for 30 min at 4°C, washed in PBS containing 20% FCS, and fixed overnight at 4°C in PBS, pH 7.2, containing 1% formaldehyde (Fisher Scientific). Hepatic MNC were isolated and 5×10^5 cells were incubated with an optimal concentrations of mAb, as described for splenic cells. Hepatic and splenic cells were similarly double labeled for the following markers: CD4/CD8, CD4/$\alpha\beta$ − TCR, LFA-1/$\alpha\beta$ − TCR, B220/$\alpha\beta$ − TCR, and CD44/$\alpha\beta$ − TCR. Isotype controls corresponding to each conjugated antibody was used.

Cytofluorometric analyses were performed on a FACScan (Becton-Dickinson, Mountain View, CA) cytofluorometer with Cell Quest software (Becton-Dickinson). Gating was performed according to forward scatter (FSC) versus 90°-angle scatter (SSC) parameters to select the distinct lymphoid cell populations. Ten thousand cells selected from this gate were analyzed per sample and percentages of various subpopulations were determined by multiparametric analysis. Experiments were conducted in triplicate or, in some experiments, data were recorded from a pool of three livers.

Blastic Tranformation

Metabolic activity of intrahepatic and splenic MNC was evaluated by the tetrazolium salt reduction test (Promega, Madison, WI). MNC were seeded at 10^6 per well (in 100-μl vol) in flat-bottom microtiter plates in RPMI 1640 containing 20% FCS, mercaptoethanol (ME), and antibiotics. Cells were treated with an optimal concentration of phytohemagglutinin A (PHA) and concanavalin A (Con A) (Sigma–Aldrich, Oakville, Ontario, Canada). After various times of incubation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS) and phenazine methosulfate (PMS) (Promega) were added and cells were further incubated for up to 4 h. The optical density was determined by using an ELISA reader (492-nm filter) (Molecular Devices, Sunnyvale, CA). The experiments were conducted in quadruplicate.

Statistical Analysis

Percentages, absolute numbers, and OD were evaluated by the Student *t* test, comparing cells from infected and control mice for *in vivo* studies or comparing infected cells to control cells in *in vitro* studies. The ratios were analyzed using the Wilcoxon–Mann–Withney U test.

RESULTS

Viral Replication in Liver from Attenuated YAC-MHV3- and Pathogenic L2-MHV3-Infected C57BL/6 Mice

We have previously demonstrated that the attenuated YAC-MHV3 variant induces a subclinical hepatitis characterized by few inflammatory foci and accompanied by perivascular infiltrates of mononuclear cells. Recovery was observed within 15 days pi (8). In order to verify the efficiency of immune-mediated elimina-

tion mechanisms of YAC-MHV3 replication, hepatic viral titers from YAC- and L2-MHV3-infected mice were compared at various times pi. In YAC-MHV3-infected mice, viral titers reached their maximum at 4 days pi ($10^{4.3 \pm 0.5}$ $TCID_{50}$/ml) and decreased within 10 days pi, while viral titers reached higher levels in pathogenic L2-MHV3-infected mice until death at 3 days pi ($10^{6.8 \pm 1.0}$ $TCID_{50}$/ml).

Analysis of CD4/CD8 Phenotypes of Intrahepatic and Splenic Lymphocytes in Attenuated YAC-MHV3- and Pathogenic L2-MHV3-Infected C57BL/6 Mice for 3 Days

To identify the intrahepatic T cell subsets involved in the control of viral infection in YAC-MHV3-infected mice, groups of three C57BL/6 mice were infected with the pathogenic L2-MHV3 or the attenuated YAC-MHV3 variant. Mice were sacrificed at 3 days pi, intrahepatic and splenic lymphoid cells were isolated, and the numbers of lymphoid cells per liver were determined by electronic counting. Numbers of intrahepatic mononuclear cells isolated from YAC-MHV3-infected mice ($1.40 \pm 0.21 \times 10^6$ cells/liver) were similar to those of uninfected mice ($1.38 \pm 0.18 \times 10^6$ cells/liver). Moreover, as previouly observed, an increase of intrahepatic mononuclear cells was observed in L2-MHV3-infected mice ($3.22 \pm 0.45 \times 10^6$ cells/liver) ($P < 0.001$) (21). To verify if splenic and intrahepatic lymphocyte subsets are modified in pathogenic L2-MHV3- or attenuated YAC-MHV3-infected mice, percentages of $CD4^+$ and $CD8^+$ T cells were phenotyped by double immunolabeling. As shown in Table 1, the percentage of $CD4^+$ cells increased in mice infected with pathogenic L2-MHV3, while it decreased in attenuated YAC-MHV3-infected mice ($P < 0.001$). However, percentages of $CD8^+$ T cells slightly increased in both infected mouse groups ($P < 0.01$). No significant differences in percentages of CD4 or CD8 cells were evidenced in spleen from both infected mice groups.

Analysis of LFA-1/$\alpha\beta$ − TCR, CD4/$\alpha\beta$ − TCR, and NK1.1/$\alpha\beta$ − TCR Phenotypes of Intrahepatic and Splenic Lymphocytes in Attenuated YAC-MHV3- and Pathogenic L2-MHV3-Infected C57BL/6 Mice for 3 Days

As previously demonstrated, the intrahepatic $\alpha\beta$ − TCR^{inter} cells expressing a high level of LFA-1 (CD11a) molecules increased in L2-MHV3-infected mice (21). To determine if the increase of LFA-$1^+\alpha\beta$ − TCR^{inter} cells was associated with viral pathogenicity, intrahepatic lymphoid cells from attenuated YAC-MHV3- and pathogenic L2-MHV3-infected mice cells were isolated at three days pi and double-labeled with FITC-anti-CD11a and PEC-anti-$\alpha\beta$ − TCR antibodies. As shown in Fig. 1A, intrahepatic $CD11a^+\alpha\beta$ − TCR^{inter} or $CD11a^-\alpha\beta$ − TCR^{inter} cell percentages decreased in YAC-MHV3-infected mice ($P < 0.05$ or $P < 0.001$), while they increased in L2-MHV3-infected mice ($P < 0.001$ or $P < 0.05$). In the spleen, however, no change was detected in the $\alpha\beta$ − TCR^{inter} subpopulations, while $CD11a^-\alpha\beta$ − TCR^{high} cell percentages decreased more in L2-MHV3- than in YAC-MHV3-infected mice ($P < 0.001$) (Fig. 1B).

Intrahepatic T cell subsets expressing an intermediate level of $\alpha\beta$ − TCR may be separated in subset groups depending on the presence of surface markers, such as CD4, CD8, or NK 1.1 (12, 13, 16, 17). To determine the phenotype of $\alpha\beta$ − TCR^{inter} cells in the livers of infected mice, double immunolabelings with FITC-anti-$\alpha\beta$ − TCR and PEC-anti-$CD4^-$ or anti-NK1.1 antibodies were performed on intrahepatic lymphoid cells isolated from pathogenic L2-MHV3- or YAC-MHV3-infected mice 3 days pi. As shown in Fig. 1C, intrahepatic $CD4^+\alpha\beta$ − TCR^{inter} cell percentages decreased in YAC-MHV3-infected mice ($P < 0.001$), while they increased in L2-MHV3-infected mice ($P < 0.05$). In addition, $CD4^+\alpha\beta$ − TCR^{high} cell percentages increased only in L2-MHV3-infected mice ($P < 0.001$). However, splenic $CD4^+\alpha\beta$ − TCR^{high} or $CD4^-\alpha\beta$ − TCR^{high} cell percentages decreased in L2-MHV3-infected mice ($P < 0.001$ or $P < 0.05$) (Fig. 1D).

On the other hand, percentages of intrahepatic $NK1.1^+\alpha\beta$ − TCR^{inter} cells decreased in both groups of infected mice ($P < 0.001$), while $NK1.1^+\alpha\beta$ − TCR^- cells were not affected (Fig. 1E). In addition, intrahepatic $NK1.1^-\alpha\beta$ − TCR^{inter} cells increased only in

TABLE 1

Percentages of $CD4^+$ and $CD8^+$ Intrahepatic and Splenic T Cells from Pathogenic L2-MHV3- and Attenuated YAC-MHV3-Infected C57BL/6 Mice at 3 Days pi

Organ	Virus	$CD4^+$	$CD8^+$
Liver	Control[a]	21.8 ± 0.7	6.7 ± 0.7
	L2-MHV3	27.1 ± 2.1*	9.0 ± 0.8**
	Control	18.4 ± 0.7	7.0 ± 1.5
	YAC-MHV3	14.1 ± 0.7*	10.0 ± 1.0**
Spleen	Control[a]	15.9 ± 2.3	6.1 ± 2.3
	L2-MHV3	15.8 ± 3.5	4.3 ± 1.8
	Control	18.8 ± 2.2	10.0 ± 2.1
	YAC-MHV3	16.7 ± 3.1	7.2 ± 1.3

[a] Intrahepatic or splenic MNC were double labeled with FITC-anti-CD4 and PEC-anti-CD8 antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and numbers of cells have been evaluated based on a total of 10,000 events recorded. Experiments were conducted in triplicate.

*$P < 0.001$; **$P < 0.05$.

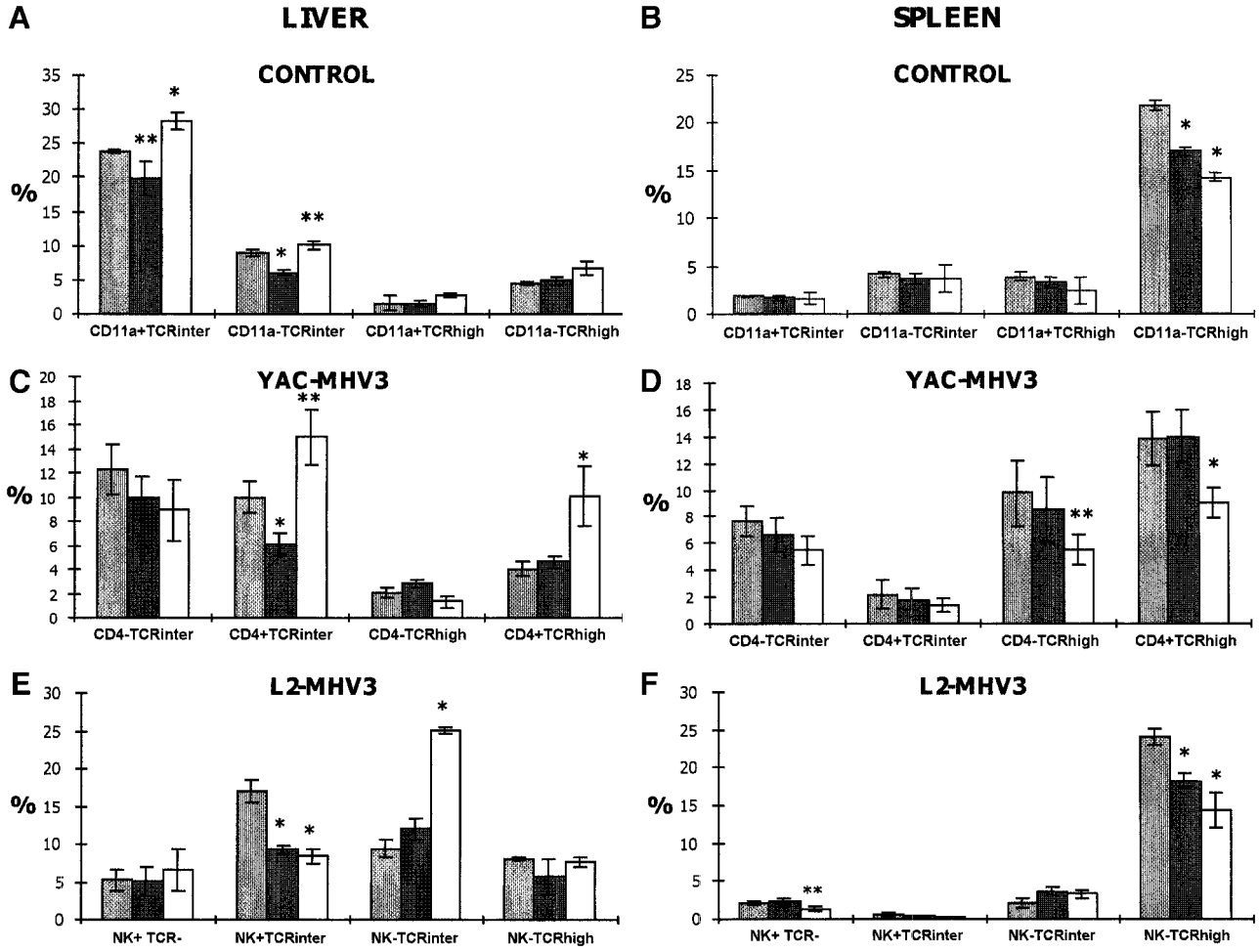

FIG. 1. Percentages of CD11a/αβ-TCR (A and B), CD4/αβ-TCR (C and D), and NK1.1/αβ-TCR (E and F) cell subsets in intrahepatic (A, C, E) and splenic (B, D, F) lymphoid cell populations, from control (□), YAC-MHV3- (■), and L2-MHV3-infected (□) C57BL/6 mice. Intrahepatic MNC were double labeled with FITC-anti-LFA-1(CD11a), FITC-anti-CD4, or FITC-anti-NK1.1 and PE-anti-αβ-TCR antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and the numbers of cells were evaluated based on a total of 10,000 events recorded. Results are representative of three experiments. $*P < 0.001$; $**P < 0.05$.

L2-MHV3-infected mice ($P < 0.001$). Splenic NK1.1$^-$αβ − TCR^{high} cell percentages decreased in both groups of infected mice ($P < 0.001$) (Fig. 1F). NK1.1$^+$αβ − TCR$^-$ slightly decreased in spleen from L2-MHV3-infected mice only ($P < 0.05$). These results suggest that an increase of intrahepatic CD4$^+$αβ − TCR^{inter} cells rather than a decrease of intrahepatic NK1.1αβ − TCR^{inter} cells correlates with viral pathogenicity.

Sequential Study of the CD4/CD8 Phenotypes of Intrahepatic Lymphocytes in YAC-MHV3-Infected C57BL/6 Mice

In order to identify the sequential evolution of intrahepatic T cell subsets favoring recovery, groups of three C57BL/6 mice were infected with the attenuated YAC-MHV3 virus and sacrificed from 24 h to 25 days pi. The number of intrahepatic MNC was recorded, and T cell subpopulations were analyzed following double immunolabeling for CD4 and CD8 markers. No significant increase in the number of intrahepatic MNC was observed during the subclinical infection for up to 25 days pi. As shown in Fig. 2, percentages of CD4$^+$ cells transiently decreased in the first 48 h pi, followed by a percentage increase up to 15 days pi. However, CD8$^+$ cells slightly decreased at 24 h pi but strongly increased for up to 25 days pi. The percentage increases of single-positive CD8$^+$ cells remained higher than those of the CD4$^+$ cells. The changes in CD4$^+$ or CD8 subpopulations were evident through CD4/CD8 ratio analysis (Table 2). The intrahepatic CD4/CD8 ratio

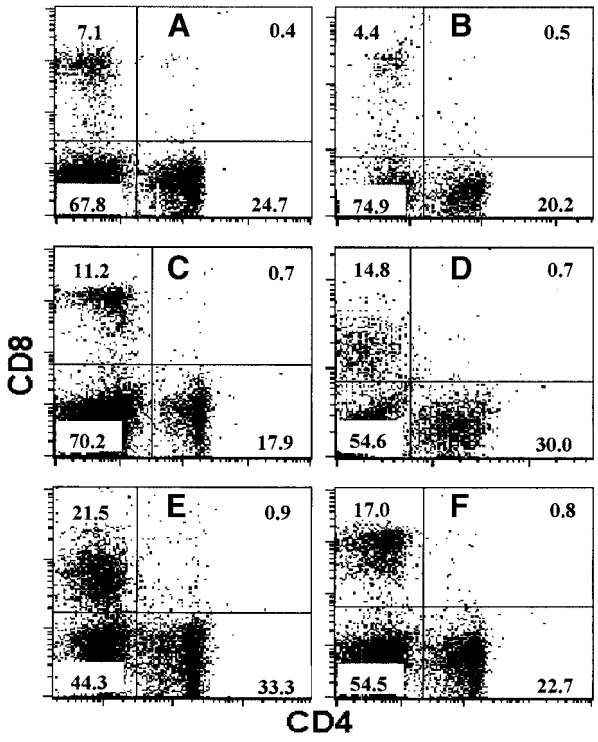

FIG. 2. Percentages of CD4 and CD8 T cells in the liver of YAC-MHV3-infected C57BL/6 mice at 0 days (A), 24 h (B), 48 h (C), 7 days (D), 15 days (E), and 25 days (F) postinfection. Intrahepatic MNC were double labeled with FITC-anti-CD4 and PE-anti-CD8 antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and the numbers of cells were evaluated based on a total of 10,000 events recorded. Results are representative of three experiments.

increased rapidly in the first 4 days pi but strongly decreased thereafter. In contrast, the CD4/CD8 ratio of splenic lymphoid subpopulations increased in the first days of infection and then returned to normal values at 15 days pi, thus indicating a longer period of $CD8^+$ cell stimulation in the liver than in spleen.

$\alpha\beta$ − TCR Profile Patterns of Hepatic $CD4^+$ Lymphocytes Isolated from YAC-MHV3-Infected C57BL/6 Mice

To verify whether the decrease of $CD4^+\alpha\beta$ − $TCR^{inter\ or\ high}$ cells observed in the attenuated YAC-MHV3-infected mice favors the subclinical infection and recovery following attenuated YAC-MHV3 infection, C57BL/6 mice were then infected with the YAC-MHV3 virus and $\alpha\beta$ − TCR profiles of $CD4^+$ cells were conducted at various times pi. As shown in Fig. 3, $CD4^+\alpha\beta$ − TCR^{high} and $CD4^+\alpha\beta$ − TCR^{inter} cell percentages decreased during the first days of viral infection. In addition, percentages of $CD4^-\alpha\beta$ − $TCR^{inter\ or\ high}$ cells increased after 7 days pi, suggesting recruitment or activation of other T cell subsets.

LFA-1 (CD11a) Patterns of Intrahepatic $\alpha\beta$ − TCR^{inter} Cells in YAC-MHV3-Infected C57BL/6 Mice

Similarly, to verify whether the decrease of the specific intrahepatic $\alpha\beta$ − $TCR^{inter}LFA\text{-}1^{high}$ cell subset favors a subclinical infection, MNC were isolated from the liver of groups of three YAC-MHV3-infected C57BL/6 mice and double immunolabeled with FITC-anti-$\alpha\beta$ − TCR and PEC-anti-LFA-1 antibodies. As observed in Fig. 4A, most intrahepatic $\alpha\beta$ − TCR^{inter} cells from control mice expressed high levels of LFA-1 ($LFA\text{-}1^+$) cells, while $\alpha\beta$ − TCR^{high} cells expressed a lower level of LFA-1 ($LFA\text{-}1^{low}$) molecules. In YAC-MHV3-infected mice, $\alpha\beta$ − $TCR^{inter}LFA\text{-}1^+$ cells transiently decreased at two days pi (Fig. 4C), while they returned to normal values thereafter (Figs. 4D–4F). On the other hand, $\alpha\beta$ − $TCR^{inter}LFA\text{-}1^{low}$ and $\alpha\beta$ − $TCR^{high}LFA\text{-}1^{low}$ cells increased in the first days pi. Also, only $\alpha\beta$ − $TCR^{inter}LFA\text{-}1^{low}$ cells remained higher until 15 days pi (Figs. 4B–4E). No major increase of $\alpha\beta$ − $TCR^{high}LFA\text{-}1^+$ cells was observed in livers from infected mice.

We have previously observed that intrahepatic $\alpha\beta$ − $TCR^{inter\ or\ high}LFA\text{-}1^+$ cells isolated from pathogenic L2-MHV3-infected mice also expressed the CD44 (PgP-1) or CD45R (B220) activation markers (21). It is postulated that infection with the attenuated YAC-MHV3 variant does not allow the activation of $\alpha\beta$ − $TCR^{inter\ or\ high}$ cell subsets. This hypothesis was verified by double immunolabeling for these activation markers of intrahepatic lymphoid cells isolated from attenuated YAC-MHV3-infected mice. No important increase of intrahepatic $CD44^+$ cells

TABLE 2

CD4/CD8 Ratios of Intrahepatic and Splenic T Cells from YAC-MHV3-Infected C57BL/6 Mice at Various Times pi

Time pi (days)	Liver	Spleen
0	3.48 ± 0.38[a]	1.70 ± 0.25
1	4.60 ± 0.22*	2.58 ± 0.27*
4	3.20 ± 0.44	2.36 ± 0.18**
7	1.35 ± 0.20*	2.91 ± 0.36*
15	1.56 ± 0.28*	1.80 ± 0.31
25	1.63 ± 0.32*	1.86 ± 0.28

[a] Intrahepatic or splenic MNC were double labeled with FITC-anti-CD4 and PE-anti-CD8 antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and numbers of cells have been evaluated based on a total of 10,000 events recorded. Experiments were conducted in triplicate.

*$P < 0.001$; **$P < 0.01$.

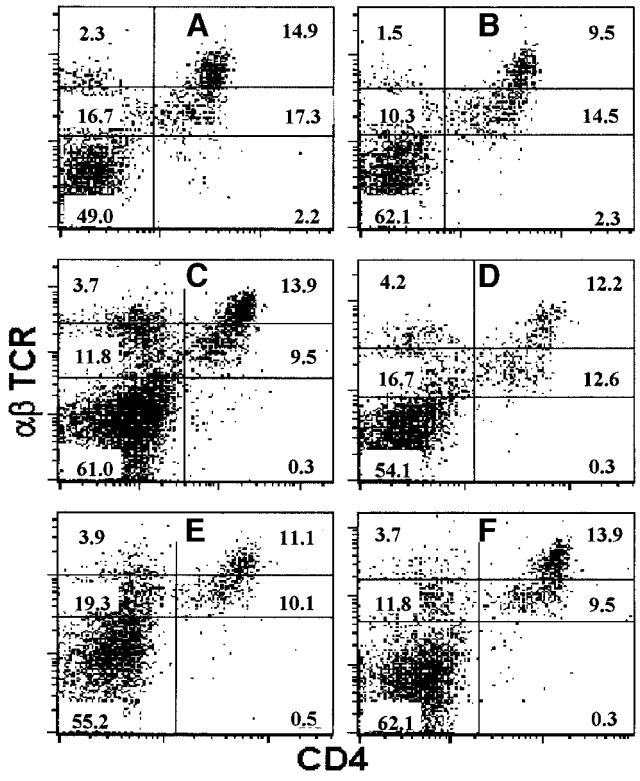

FIG. 3. Percentages of CD4$^+\alpha\beta$-TCR^{inter} and CD4$^+\alpha\beta$-TCR^{high} T cells in the liver of YAC-MHV3-infected C57BL/6 mice at 0 days (A), 24 h (B), 48 h (C), 4 days (D), 6 days (E), and 25 days (F) postinfection. Intrahepatic MNC were double labeled with FITC-anti-CD4 and PE-anti-$\alpha\beta$-TCR antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and the numbers of cells were evaluated based on a total of 10,000 events recorded. Results are representative of three experiments.

was observed in YAC-MHV3-infected mice, except for intrahepatic CD44+$\alpha\beta$ − TCR^{high} cells (5.1 ± 0.5%) ($P < 0.05$) when compared with control mice (3.3 ± 0.6%), while splenic $\alpha\beta$ − TCR^{inter}CD44$^+$ cells slightly decreased (YAC-MHV3-infected mice, 3.5 ± 1.3%; control mice, 6.3 ± 1.3%) ($P < 0.05$). Other than a decrease in $\alpha\beta$ − TCR^{inter}B220$^-$ cells (YAC-MHV3-infected mice, 3.9 ± 0.4%; control mice, 8.9 ± 2.0%) ($P < 0.001$), no differences were observed in percentages of hepatic or splenic $\alpha\beta$ − TCR$^{high\ or\ inter}$B220$^+$ cells. In addition, no significant differences were found in intrahepatic or splenic total CD44$^+$ or B220$^+$ subsets from 3 to 25 days pi (results not shown).

Ex vivo Blastic Transformation of Intrahepatic and Splenic Lymphoid Cells from L2-MHV3- and YAC-MHV3-Infected C57BL/6 Mice

In order to verify whether intrahepatic MNC from L2-MHV3- and YAC-MHV3-infected mice were able to respond to antigenic stimulation, intrahepatic and splenic lymphoid cells were isolated from L2-MHV3- and YAC-MHV3-infected mice at 3 days pi and from YAC-MHV3-infected mice at 14 days pi. The cells were stimulated with PHA or Con A, and the level of blastic transformation was evaluated by a colorimetric method based on tetrazolium salt reduction. Results shown in Fig. 5 indicate that intrahepatic lymphoid cells isolated from L2-MHV3-infected mice were totally anergic 3 days pi, while those from YAC-MHV3-infected mice were less activated than cells from uninfected mice (Fig. 5A) ($P < 0.001$). At 14 days pi, cell activation by Con A or PHA was greater in intrahepatic lymphoid cells of YAC-MHV3-infected mice than in those of the control group (Fig. 5A) ($P < 0.001$). However, splenic lymphoid cells from YAC-MHV3- or L2-MHV3-infected mice at 3 or 14 days retained their ability to respond to Con A and PHA stimulation, but activation levels of splenic cells were higher in cells from YAC-MHV3-infected mice at three days pi ($P < 0.001$ and $P < 0.05$) (Fig. 5B).

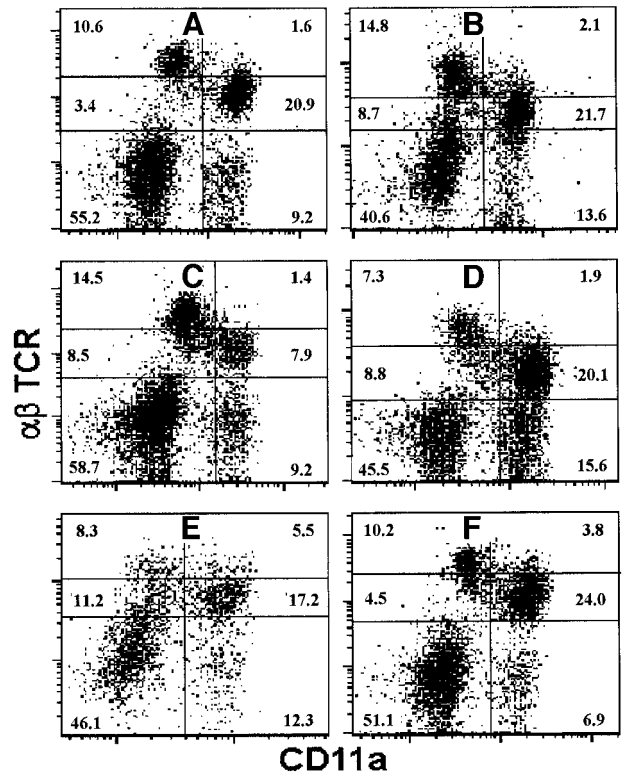

FIG. 4. Percentages of LFA-1$^+\alpha\beta$-TCR^{inter}, LFA-1$^+\alpha\beta$-TCR^{high}, LFA-1$^{high}\alpha\beta$-TCR^{inter}, and LFA-1$^{high}\alpha\beta$-TCR^{high} T cells in the liver of YAC-MHV3-infected C57BL/6 mice at 0 days (A), 24 h (B), 48 h (C), 4 days (D), 6 days (E), and 25 days (F) postinfection. Intrahepatic MNC were double labeled with FITC-anti-LFA-1(CD11a) and PE-anti-$\alpha\beta$-TCR antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and numbers of cells were evaluated based on a total of 10,000 events recorded. Results are representative of three experiments.

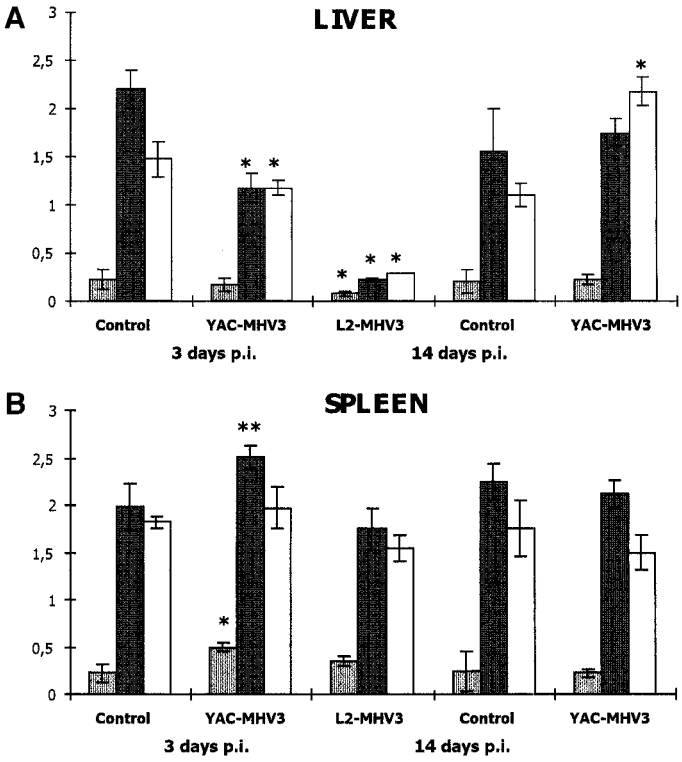

FIG. 5. Blastic transformation of intrahepatic (A) and splenic (B) lymphoid cells isolated from L2-MHV3- and YAC-MHV3-infected or control mice at 3 and 14 days pi, *in vitro* unstimulated (▨) and stimulated with PHA (■) or Con A (□). Metabolic activity of intrahepatic and splenic MNC was evaluated using the MTS reduction test. After 72 h of incubation, MTS and PMS (Promega) were added and cells were further incubated for up to 4 h. The optical density was then recorded using an ELISA reader (492-nm filter). The experiments were conducted in quadruplicate. $*P < 0.001$; $**P < 0.05$.

Phenotype of Inactivated Intrahepatic T Cell Subset in YAC-MHV3- or L2-MHV3-Infected Mice

To determine the phenotype of partially or totally anergized intrahepatic lymphoid cells of YAC-MHV3- and L2-MHV3-infected mice, respectively, lymphoid cells were isolated from the liver and spleen of both groups of virus-infected mice at 3 days pi. Subsequently, $CD8^+$ cells were depleted by cytotoxicity using anti-CD8 antibodies and complement. CD4/CD8 double immunolabeling of residual cells revealed that less than $1.66 \pm 0.03\%$ expressed the CD8 marker. Cells were then cultured for 71 h, and the metabolic activity of residual T cells was evaluated by reduction levels of tetrazolium salts. As shown in Fig. 6A, metabolic activity of intrahepatic T cells from control mice partially decreased when $CD8^+$ T cells were removed ($P < 0.05$). However, the decrease was exacerbated in CD8-depleted intrahepatic cells from YAC-MHV3-infected mice ($P < 0.001$) (Fig. 6B). In addition, metabolic activities of intrahepatic lymphoid cells before and after CD8 depletion were depressed in L2-MHV3-infected mice ($P < 0.001$) (Fig. 6C). However, such decreases after removal of $CD8^+$ cells were less evident in splenic cells isolated from both groups of infected mice (Figs. 6D–6F). These results indicate that $CD8^+$ cell subsets contributed mainly to the metabolic activity of total intrahepatic lymphoid cells isolated from YAC-MHV3-infected mice. Hence, only $CD4^+$ cell subsets were anergized in YAC-MHV3-infected mice, while both $CD8^+$ and $CD4^+$ subsets were anergized in L2-MHV3-infected mice.

Modulation of Intrahepatic $CD4^+$ Cell Subsets by in Vitro L2-MHV3 and YAC-MHV3

To determine the role of viral infection in the modulation of intrahepatic CD4 subsets, as observed in L2-MHV3- and YAC-MHV3-infected mice, intrahepatic lymphoid cells were isolated and infected *in vitro* with both viruses for 24 h. CD4 subsets were discriminated by a double immunolabeling with FITC-anti-$\alpha\beta$ − TCR and PEC-anti-CD4 antibodies. As shown in Fig. 7, the YAC-MHV3 or the L2-MHV3 virus did not induce significant alterations in CD4 subsets in *in vitro* infected intrahepatic lymphoid cells. However, when $CD8^+$ cells were depleted by cytotoxicity with anti-CD8 antibodies and complement, $\alpha\beta - TCR^{inter}CD4^+$ cell percentages increased in YAC-MHV3-infected cells ($P < 0.05$) (Fig. 7B). Such increases were higher in L2-MHV3-infected cells after $CD8^+$ cell depletion ($P < 0.001$). In addition, $CD4^+\alpha\beta - TCR^{high}$ cells slightly increased in YAC-MHV3-infected cells, while they decreased in L2-MHV3-infected cells ($P < 0.05$). Analysis of absolute numbers of such T cell subsets revealed that the relative percentage increase of $CD4^+\alpha\beta - TCR^{high}$ cells observed in control and infected cells reflected the depletion of $CD8^+\alpha\beta - TCR^{high}$ cells rather than a specific increase of $CD4^+\alpha\beta - TCR^{high}$ cells (results not shown).

DISCUSSION

In this paper, we report that the subclinical hepatitis induced by the attenuated YAC-MHV3 variant is related to low activation of intrahepatic $CD4^+$ subsets, despite the presence of viral replication in the liver during the first days of infection, and to the recruitment of $CD8^+$ cells, favoring viral elimination within 10 days pi.

Suppression of intrahepatic $CD4^+\alpha\beta - TCR^{inter}$ cells from C57BL/6 mice infected with the attenuated YAC-MHV3 virus is supported by many observations. First, intrahepatic lymphoid cells did not increase in YAC-MHV3-infected mice, in contrast to the increase ob-

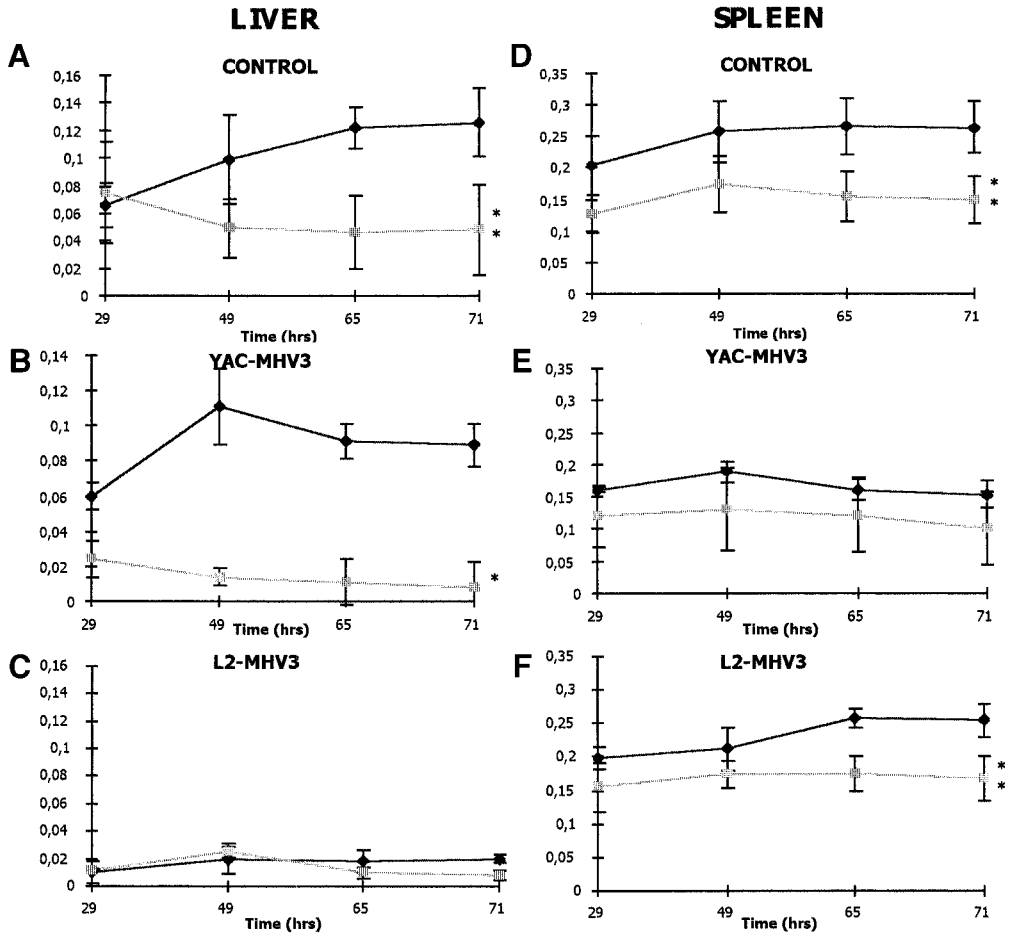

FIG. 6. Metabolic activity of intrahepatic (A–C) and splenic (D–F) lymphoid cells isolated from L2-MHV3- (C and F) and YAC-MHV3- (B and E) infected mice or control mice (A and D) at three days pi, *in vitro* untreated (◆) and treated (□) with anti-CD8 antibodies and complement for 24 h. Metabolic activity of intrahepatic and splenic MNC was evaluated using the MTS reduction test. After various times of incubation, MTS and PMS (Promega) were added and cells were further incubated for up to 4 h. The optical density was then recorded using an ELISA reader (492-nm filter). The experiments were conducted in quadruplicate. $*P < 0.001$; $**P < 0.05$.

served in pathogenic L2-MHV3-infected mice (21). Second, percentages of intrahepatic CD4$^+\alpha\beta$ − TCR^{inter} and CD4$^+\alpha\beta$ − TCR^{high} cells decreased in YAC-MHV3-infected mice, while they increased in pathogenic L2-MHV3-infected mice. Moreover, TCR$^{inter\ or\ high}$LFA-1^{high} subsets did not increase during the first days of infection with the YAC-MHV3 virus, as observed in L2-MHV3-infected mice (21). In addition, B220$^+$ or CD44$^+$ T cell subsets normally found in actively proliferating intrahepatic lymphoid cells (13, 16, 22) did not increase in attenuated YAC-MHV3-infected mice. Finally, CD8-depleted intrahepatic T lymphoid cells isolated from YAC-MHV3-infected mice could not be stimulated by lectins.

The role of anergized intrahepatic CD4$^+$ cell subsets in the hepatitis process may be evaluated in regard to the phenotypic changes of intrahepatic lymphoid cells in pathogenic L2-MHV3-infected mice. The decrease of intrahepatic NK-T cell subsets in both groups of infected mice suggests that this cell subset does not play a major role in viral elimination. We have previously shown that intrahepatic CD4$^+$TCR$^{inter\ or\ high}$LFA-1^{high} increased in acute hepatitis induced by the pathogenic L2-MHV3 virus (21). In this work, we demonstrated that, in attenuated YAC-MHV3-infected mice, intrahepatic CD4$^+$TCR^{inter}LFA-1^{high} cells transiently decreased. In addition, these cells were anergized, since the partial stimulation of intrahepatic lymphoid cells from YAC-MHV3-infected mice was mainly due to CD8$^+$ cells, rather than CD4$^+$ cell subsets. Such a phenomenon did not occur in the spleen, suggesting that a specific intrahepatic mechanism occurs in the first days of infection, leading to a blockade of the maturation process or induction of a suppressive state of intrahepatic CD4$^+$TCR^{inter} cells comparable to that seen in immune tolerance.

Immune tolerance may be due to the unresponsiveness of resident T lymphocytes despite antigen pre-

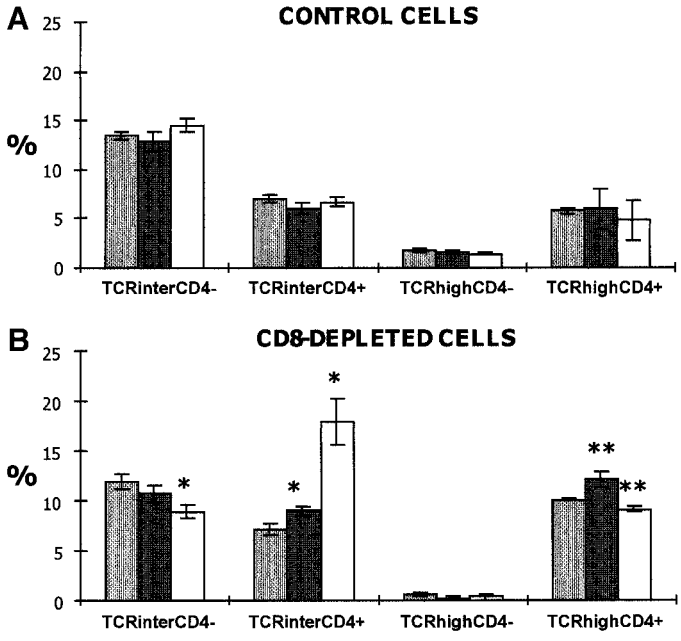

FIG. 7. Percentages of CD4/$\alpha\beta$-TCR subsets in *in vitro* untreated (A) and CD8-depleted (B) intrahepatic lymphoid cells, subsequently uninfected (▨) or infected with L2-MHV3 (□) and YAC-MHV3 (■) viruses. Intrahepatic cells were isolated from C57BL/6 mice, treated with anti-CD8 antibodies and complement, infected with 0.1 to 1 m.o.i. of each virus, and then incubated for 24 h. Cells were then double labeled with FITC-anti-CD4 and PEC-anti-$\alpha\beta$-TCR antibodies and analyzed using a FACScan cytofluorometer. Lymphoid cells were gated according to FSC/SSC parameters, and numbers of cells were evaluated based on a total of 10,000 events recorded. Results are representative of three experiments. $^{*}P < 0.001$; $^{**}P < 0.05$.

sentation and activating cytokine production. The unresponsiveness of intrahepatic lymphocytes may result from several mechanisms. It has been proposed that an autocrine loop initiated by an interaction beween lymphocytes and Kupffer cells can cause $CD4^+$ activation and IFN-γ production, which in turn may induce NO release by Kupffer cells to suppress T cell proliferation (23, 24). Kupffer cells are an important target for pathogenic L2-MHV3 replication in the liver (25, 26), suggesting that this proposed mechanism would be altered during viral infection. YAC-MHV3 replication was lower in macrophages than that produced by pathogenic L2-MHV3 infection (27), suggesting that the tolerance, which is under control by Kupffer cells, would not be disturbed in YAC-MHV3-infected mice. Moreover, the production of IFN-$\alpha\beta$, tumor necrosis factor-α, or prostaglandin E2 (PGE2) by Kupffer cells could also be responsible for the decreased proliferative response of hepatic T cells (23, 24). *In vivo* treatment of attenuated YAC-MHV3-infected mice with a PGE2 inhibitor, such as indomethacine, did not increase the hepatitis severity and percentages of intrahepatic activated $CD4^+TCR^{inter\ or\ high}LFA\text{-}1^{high}$ cells (results not shown), suggesting that suppression of those lymphocytes is not under the control of PGE2. In addition, NK-T cells are known to be high producers of IL-4, favoring the stimulation of Th2 cells and production of cytokines involved in suppression of the Th1 response, such as that seen in immune tolerance (28). Since NK-T cells decreased in liver from both infected groups of mice, it is speculated that a decrease of Th2-dependent cytokines favors the activation of Th1 cells in both groups of infected mice. However, this mechanism may partially explain the activation of intrahepatic T cell subsets observed in L2-MHV3-infected mice (21) but cannot support the decrease of $CD4^+$ cell subsets in YAC-MHV3-infected mice. In addition, the $CD4^+$ belonging to the Th1 phenotype or NK cells can produce IFN-γ, which in turn is able to control viral replication. Preliminary *in vitro* experiments suggested that, in contrast to the L2-MHV3 virus, the YAC-MHV3 variant cannot replicate in the presence of IFN-γ.

The liver could be an elimination site for activated lymphocytes, as suggested by an increase of apoptotic liver-resident lymphocytes following antigenic stimulation or by their localization in hepatic sinusoids or in foci of degenerative hepatocytes caused by lymphocyte–thrombi-induced ischemia (22). No significant increase of apoptotic cells has been detected by a TUNEL test in intrahepatic lymphoid cells isolated from YAC-MHV3-infected mice (results not shown), suggesting that anergy observed in CD8-depleted cells, as soon as 3 days pi, was due not to an increase of activated lymphoid cells recruited from peripheral immune organs, but to an *in situ* cytokine-deprived suppression.

In contrast with other organs, leukocyte adhesion to intrahepatic endothelial cells (EC) does not require the expression of selectins but depends upon the endothelial expression of vascular adhesion protein 1 (29), LFA-1(CD11a)-CD54, and CD106-CD49 surface molecules (30). In addition, the number of leucocytes adhering to intrahepatic EC is controlled by a leucocyte–EC interactions and remains constant even if the total number or circulating leukocytes varies over time (30). However, EC are an important target for pathogenic L2-MHV3 replication but not for attenuated viral strains (25, 26). Other cytokines, such as IL-10 and prostanoids released by Kupffer cells, downregulate leukocyte adhesion to intrahepatic EC by decreasing surface expression of CD54 and CD116 (29) and then avoiding the recruitment of lymphoid cells and the immune-mediated liver damage by antigens from portal venous blood (31, 32). Our observations concerning the numbers of intrahepatic lymphoid cells from attenuated YAC-MHV3-infected mice support the hypothesis that the integrity of intrahepatic EC is essential to avoid recrutment and/or activation of T lymphocytes involved in the hepatitis process.

On the other hand, the higher expression of LFA-1 molecules on intrahepatic TCR^{inter} cells may favor the binding of lymphocytes to some hepatic cells expressing a high level of ICAM-1. It is speculated that suppression of $CD4^+TCR^{inter}LFA\text{-}1^{high}$ cells, as seen in YAC-MHV3-infected mice, favors a down-regulation of ICAM-1 expression in hepatocytes in impairing production of cytokines by these cells, thus avoiding the binding of cytotoxic cells to hepatocytes. The decrease in T cells expressing high levels of LFA-1 would also be protective against destruction of the EC barrier by avoiding exposure of the underlying hepatocytes to further attack by cytotoxic cells. This hypothesis is supported by the fact that intrahepatic T cell subsets cannot be stimulated by mitogens and that intrahepatic lymphoid cells from attenuated YAC-MHV3-infected mice did not express activation markers, such as CD44 or CD45R (B220). This mechanism has been previouly demonstrated in the model of Con A-induced hepatitis in which the fulminant disease was mediated by activated $CD4^+$ lymphocytes following their contact with hepatocyte-bound Con A (33, 34). In this situation, hepatic tolerance may be broken and tissue damages may result from the action of T cells activated by a liver microenvironment that has previously been conditioned by a microbial infection.

In this work, we have also observed that intrahepatic $CD8^+$ cells are not anergic and may act as protective cells favoring viral elimination. Such a protective role of intrahepatic CD8 T cells has been reported in the hepatitis induced by *Listeria monocytogenes*, in which adoptive transfer of splenic $CD4^+$ T cells alone exacerbated the disease, while bacterial clearance was mediated by cytotoxic $CD8^+$ cells (35). The percentage increase of intrahepatic $CD8^+$ T cells observed in the YAC-MHV3-infected mice may result either from an *in situ* stimulation of intrahepatic resident $CD8^+$ cells or from a recruitment of circulating lymphocytes. The fact that percentages of $CD8^+$ T cells transiently decreased in the liver while splenic $CD8^+$ cells increased indicates that a peripheral stimulation of splenic $CD8^+$ cells occurs before the increase of intrahepatic $CD8^+$ cells. The increase of intrahepatic T cells subsets expressing a lower level of LFA-1 antigen, such as that observed in splenic cells (14, 21), suggests that intrahepatic $CD8^+$ cells are recruited from the blood. However, the recruitment of $CD8^+$ cells requires that there may be no major intrahepatic vascular disorders induced by the viral infection, such as demonstrated in pathogenic L2-MHV3 infection (36). We can thus postulate that attenuated YAC-MHV3 infection in liver does not involve intravascular coagulation, as demonstrated by nonextensive pathological damages with perivascular lymphoid cells (8), thus enabling the recruitment of circulating activated $CD8^+$ T cells. This hypothesis is also supported by the observation of lower levels of $CD8^+$ cells and the strong anergy observed in *ex vivo* lectin-stimulated intrahepatic lymphoid cells from L2-MHV3-infected mice.

Interestingly, *in vitro* L2-MHV3 infection of intrahepatic lymphoid cells revealed that intrahepatic $CD4^+TCR^{inter\ or\ high}$ cell subsets increased when $CD8^+$ cells were depleted, suggesting that $CD8^+$ cells may prevent the activation of CD4 cell subsets. However, depletion of $CD8^+$ cells in YAC-MHV3-infected intrahepatic lymphoid cells did not provoke a strong increase of $CD4^+$ subsets, indicating that an increase of CD4 cell subsets depends on both the pathogenic properties of the virus strain and the absence of $CD8^+$ cells.

We can conclude that the attenuated phenotype of the YAC-MHV3 variant is related to recruitment of cytototoxic $CD8^+$ cells produced in peripheral lymphoid organs, such as the spleen, allowing the elimination of infected cells in the liver without breaking hepatic immune tolerance mechanisms. Further work is in progress to identify the virally mediated cell dysfunctions involved in the breakdown of tolerance mechanisms induced during the L2-MHV3 infection and in the recruitment of peripheral $CD8^+$ cells in YAC-MHV3-infected mice.

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