Telbivudine preserves T-helper 1 cytokine production and downregulates programmed death ligand 1 in a mouse model of viral hepatitis

Z. G. Wu,[1] W. M. Yan,[1] W. Guo,[1] T. Chen,[1] Y. Zou,[1] H. W. Wang,[1] X. J. Wang,[1] X. J. Yang,[1] Y. L. Lu,[1] X. P. Luo[2] and Q. Ning[1]   [1]*Department of Infectious Disease, Institute of Infectious Disease; and* [2]*Department of Pediatrics, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

SUMMARY. Telbivudine is an orally bioavailable L-nucleoside with potent and specific anti-hepatitis B virus activity. The higher rate of hepatitis B e antigen (HBeAg) seroconversion during telbivudine treatment than other potent anti-HBV agents suggests a potential immunomodulatory effect. We sought to determine the effects of telbivudine on the immune system, particularly on cytokine production and T-cell response, using an animal model with mouse hepatitis virus strain 3 (MHV-3)-induced hepatitis. The effects of telbivudine on virus replication and cytokine production were investigated *in vitro* using MHV-3-infected macrophages, and the effects on T-cell response were investigated *in vivo* in an MHV-3-induced viral hepatitis model. Telbivudine had no effect on MHV-3 replication in macrophages. However, the production of tumour necrosis factor-α and interleukin-12 was increased significantly in MHV-3-induced macrophages treated with telbivudine. *In vivo* survival was enhanced in telbivudine-treated mice, with marked normalization in clinical conditions and histological lesions. Serum levels of interferon-γ were elevated significantly after telbivudine treatment in MHV-3-infected C3H mice. In contrast, serum interleukin-4 levels were decreased significantly. Furthermore, telbivudine treatment enhanced the ability of T cells to undergo proliferation and secrete cytokines but did not affect cytotoxicity of infected hepatocytes. Of note, we found that telbivudine treatment suppressed programmed death ligand 1 expression on T cells. The results demonstrate the immunomodulatory properties of telbivudine, independent of its antiviral activity, in a mouse model of MHV-3-induced hepatitis.

Keywords: hepatitis B virus, interleukin, oral nucleos(t)ide analogue, programmed death ligand 1, tumour necrosis factor.

INTRODUCTION

Hepatitis B virus (HBV) infection is a significant cause of liver-related morbidity and mortality, particularly in regions of high endemicity [1]. The natural course and clinical outcome of HBV infection is mediated by a complex interaction between the virus and the host's immune response. Successful control of HBV replication is associated with a strong, multi-specific CD4^{+} and CD8^{+} T-cell response in conjunction with a humoral immune response [2–5]. Based on current understanding of host–virus interactions and the natural course of HBV infection, management strategies have focussed on enhancement of the host's HBV-specific T-cell response and direct suppression of HBV replication to attain sustained viral control and remission of liver disease [6,7]. Of the currently available anti-HBV therapies, only interferon (IFN)-α and pegylated IFN-α have demonstrated an immunomodulatory effect on HBV [8]. The immunomodulatory effect has been reported with the nucleoside ribavirin; the mechanisms of action are considered to be Th1 cell differentiation and upregulation of activity of double-stranded (ds) RNA-activated protein kinase [9–11]. Very little is known about the influence of newer oral nucleosides on the T-cell-specific immune response.

Telbivudine, an L-nucleoside analogue of thymidine, is a potent and specific inhibitor of HBV DNA polymerase, and it is approved for the treatment of patients with chronic hepatitis B (CHB) [12–16]. In the 2-year analysis of the GLOBE

Abbreviations: CHB, chronic hepatitis B; FasL, Fas ligand; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; MHV-3, mouse hepatitis virus strain 3; PD-1, programmed death 1; PD-L1, programmed death ligand 1.

Correspondence: Dr Qin Ning, Department of Infectious Disease, Institute of Infectious Disease, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, 1095 Jie Fang Avenue, Wuhan 430030, China. E-mail: qning@tjh.tjmu.edu.cn

study, the rate of hepatitis B e antigen (HBeAg) seroconversion during telbivudine administration was higher than during lamivudine administration in patients with serum alanine aminotransferase (ALT) levels greater than two times the upper limit of normal (ULN) [17]. The relatively high HBeAg seroconversion rate attained with telbivudine differs from the rates obtained with other potent anti-HBV agents and suggests that telbivudine potentially has immunomodulatory activities.

The aims of the present study were to characterize the effects of telbivudine on cytokine profile and T-cell response *in vitro* and *in vivo* using a previously characterized mouse model of viral hepatitis induced by the coronavirus mouse hepatitis virus strain 3 (MHV-3) [18,19]. The effect of telbivudine on the programmed cell death pathway, a possible mechanism by which CD8$^+$ T-cell function was influenced, was also investigated.

MATERIALS AND METHODS

Telbivudine and lamivudine

Telbivudine (pure substance) was obtained from Novartis Pharma (Basel, Switzerland) and lamivudine was purchased from Moravek Biochemical (Brea, CA, USA). Substance concentrations were determined by high-performance liquid chromatography.

Virus

Mouse hepatitis virus strain 3 was originally obtained from the American Type Culture Collection (Rockville, MD, USA) and it was plaque purified on monolayers of DBT cells and grown to titres of 2×10^7 plaque-forming units (p.f.u.)/mL. Virus was harvested by centrifugation at 4500 g for 1 h at 4 °C and was assayed on monolayers of L2 cells in a standard plaque assay.

Mice

Female BALB/cJ and C3H mice at 6–8 weeks of age were obtained from the Hubei Provincial Institute of Science and Technology (Wuhan, China) and the Vital River Company (Beijing, China), respectively. All animal studies were conducted in accordance with the Chinese Council on Animal Care and they were approved by Tongji Hospital (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China).

In vitro *study*

Macrophage isolation
Peritoneal macrophages were harvested from BALB/cJ mice 4 days after an intraperitoneal injection of 1.5 mL of 3% thioglycollate, as outlined previously [20]. Macrophages were ≥95% pure, as determined by morphology. Viability exceeded 95% by Trypan blue exclusion.

Effect of telbivudine on the replication of MHV-3 in macrophages
Macrophages were incubated in the presence of indicated concentrations of telbivudine (10, 50 or 100 μg/mL) with MHV-3. Macrophages were harvested 10 h after infection and viral titre was determined in a standard plaque assay.

Cytokine level measurement by PCR and enzyme-linked immunosorbent assay
To determine the effect of telbivudine on tumour necrosis factor (TNF)-α and interleukin (IL)-12 cytokine production, one million macrophages from BALB/cJ mice were stimulated with MHV-3 (multiplicity of infection, 2.5) and telbivudine was added at indicated concentrations. Relative quantitative real-time polymerase chain reaction (PCR) was performed to measure the mRNA levels on a Roche Light-Cycler$^\circledR$ (Roche Diagnostics, Nutley, NJ, USA) following the manufacturers' instructions. Cytokine protein levels were determined in cell supernatants following appropriate dilution using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). The cytokine levels in lipopolysaccharide (LPS)-stimulated macrophages with or without telbivudine added from the same strain of mice were also investigated.

In vivo *study*

Animal model
Fifteen C3H mice each infected with 10 p.f.u. MHV-3 received telbivudine 60 mg/kg in 100 μL phosphate-buffered saline (PBS) via intraperitoneal injection for 15 days, starting 2 h before infection and daily thereafter. An additional 15 MHV-3-infected mice in each group received lamivudine 60 mg/kg per day or PBS as a control. Clinical conditions and survival were observed. Animals were killed on Day 15 after infection, and spleens, livers and blood were collected for experiments.

Clinical conditions
The effect of treatment with telbivudine on MHV-3-induced liver disease was determined by monitoring mice twice daily for changes in clinical signs, including abnormalities of fur texture, increased respiration, and the presence of tremor. Conditions were graded on a scale of 1–5, with 1 indicating the marked presence of an abnormality and 5 indicating normalcy. A composite score for all parameters was calculated by two independent examiners who were blinded to the treatment regimens to minimize bias.

Liver histology
At the time the mice were killed, livers were removed, weighed and fixed in 10% buffered formalin for a minimum of 4 h. Tissues were processed and paraffin embedded by

routine methods, and they were stained with haematoxylin and eosin.

Isolation of splenocytes and CD8+ T cells

Spleens from each group of mice were harvested 15 days after MHV-3 infection. Spleens were dissected and single-cell suspensions were made by passage through a nylon cell strainer. After density gradient centrifugation and lysis of red cells in ammonium chloride solution, cells were prepared for study. CD8+ T cells were purified from single-cell splenocyte suspension using an isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Hepatocytes

Mouse hepatitis virus strain 3-infected C3H mice were anaesthetized and their livers were perfused *in situ* with a Ca^{2+}-free Hank's balanced salt solution containing 1 mM ethylenediaminetetraacetic acid for 4 min at 37 °C, followed by a 0.05% collagenase solution (type IV; Sigma-Aldrich, St Louis, MO, USA) for 10 min. After digestion, the livers were removed and placed into a sterile Petri dish. Single-cell suspensions were prepared. The viability of hepatocytes both before and after treatment was ≥95%, as assessed by Trypan blue staining.

Serum collection and cytokine measurements

On the day the mice were killed, sera were collected and assayed for IL-4 and IFN-γ using the appropriate ELISA (R&D Systems).

Intracellular staining for cytokines

Mice were sacrificed at the indicated time points. Purified cells were cultured at 2×10^6 cells/mL in the presence of 25 ng/mL phorbol myristate acetate plus 1 μg/mL ionomycin for 5 h in 24-well plates. Cells were then stained with monoclonal antibodies specific for surface markers, permeabilized, and fixed using Cytofix/Cytoperm™ (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Different cell subsets were identified using antibodies to IL-4, IL-10, IFN-γ, TNF-α, perforin, granzyme B (eBioscience, San Diego, CA, USA) and their isotypes.

Flow cytometry

The antibodies used for staining were anti-CD3 (PE-Cy5.5), anti-CD8 (APC), anti-CD4 [fluorescein isothiocyanate (FITC)], anti-programmed death 1(PD-1), anti-programmed death ligand 1 (PDL-1), anti-Fas ligand (FasL), anti-perforin, anti-granzyme B, anti-TNF-α-R1/2, anti-TNF-α, anti-IFN-γ (PE), and isotype control (PE) (eBioscience). All analyses were performed on a FACSAria™ cell sorter (BD), and subsequent analysis was performed with FACSDiva software (BD).

Cytotoxicity against hepatocytes

Freshly isolated hepatocytes from MHV-3-infected C3H mice were used as target cells and CD8+ T cells as effector cells.

Cytolytic activity was determined with the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI, USA). For each effector:target cell ratio (ranging from 40.0:1 to 2.5:1), specific target cell lysis was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Each effector:target cell ratio was measured in quadruplicate.

Statistical analysis

Statistical analysis was conducted with a Student's *t*-test, and a *P*-value of ≤0.05 was considered statistically significant. Results were reported as the mean ± standard deviation for three or more separate experiments, each performed in triplicate.

RESULTS

Telbivudine does not affect MHV-3 replication in macrophages

First, we investigated whether telbivudine has no cytotoxic effect on macrophages. The addition of up to 100 μg/mL telbivudine to peritoneal macrophages freshly isolated from BALB/cJ mice had no toxic effects, as demonstrated by Trypan blue exclusion (95% viable cells).

To examine the efficacy of telbivudine on viral replication directly, isolated macrophages were treated with telbivudine at indicated concentrations. The starting concentration (10 μg/mL) was chosen based on maximal concentration and area under the concentration–time curve from zero to infinity, and it was as close to twice the dose as that for patient administration [21,22]. Telbivudine had no effect on MHV-3 replication from the starting dose of 10 μg/mL to doses as high as 50 and 100 μg/mL (Fig. 1).
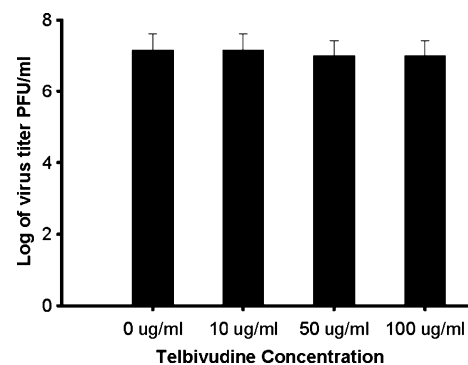

Fig. 1 Telbivudine does not affect MHV-3 replication in BALB/cJ macrophages *in vitro*. Confluent monolayers of macrophages were infected with 2×10^6 MHV-3 in the presence of indicated concentrations of telbivudine for 10 h. Viral titres were measured on monolayers of L2 cells. Data are presented as mean ± SD for three separate experiments done in duplicate.

Telbivudine enhanced the production of TNF-α and IL-12 in macrophages after MHV-3 infection but not in LPS-induced macrophages

Experiments were performed to test the ability of telbivudine to modulate the immune response of MHV-3-infected macrophages. Macrophages produced significantly higher levels of TNF-α and IL-12 in response to MHV-3 infection in comparison with basal values. Telbivudine significantly enhanced the production of TNF-α and IL-12 in MHV-3-infected macrophages compared with controls and this effect occurred in a dose-dependent manner (Figs 2b,d). A similar pattern of increase in TNF-α and IL-12 mRNA production was also observed in response to telbivudine (Figs 2a,c). However, telbivudine had no effect on TNF-α and IL-12 levels or mRNA production in LPS-stimulated macrophages (Figs 2a,b).
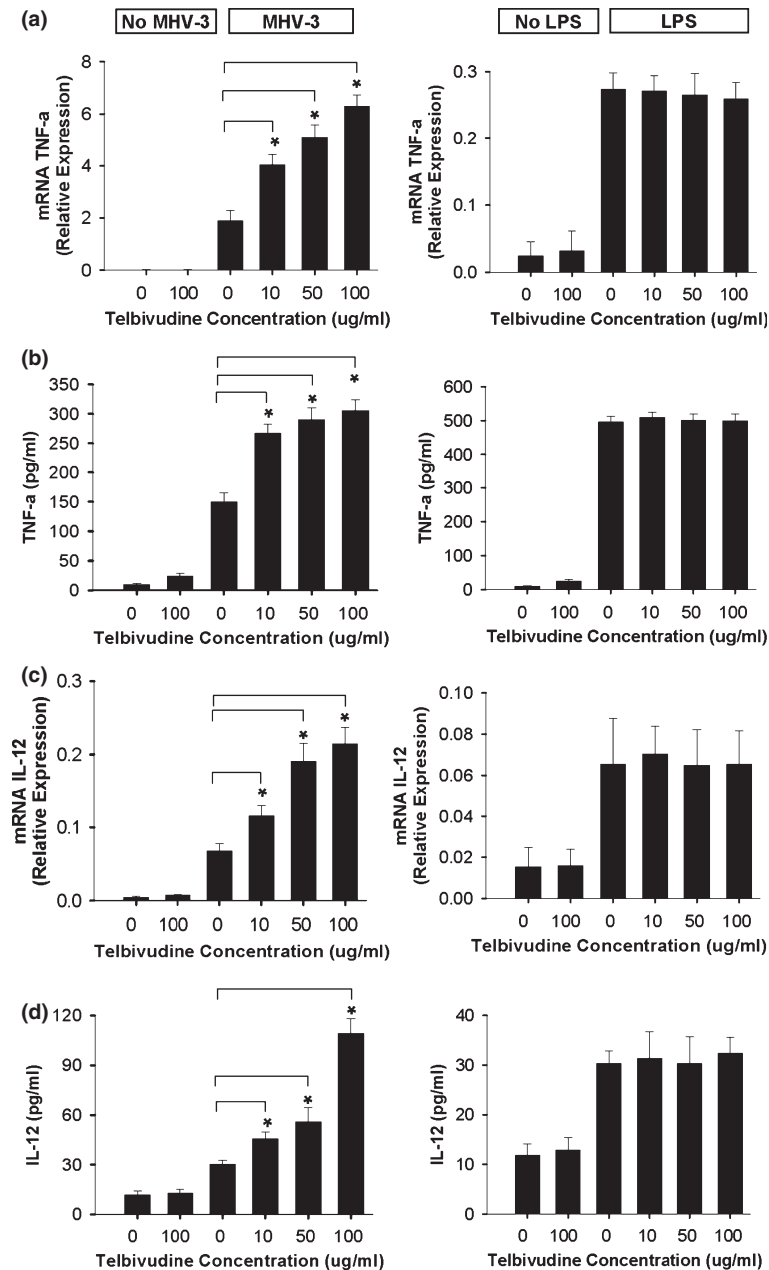

Fig. 2 Effect of telbivudine on MHV-3-infected macrophages *in vitro*. The left panels show that telbivudine enhanced the production and mRNA transcription of TNF-α (a,b) and IL-12 (c,d) in macrophages after MHV-3 infection. The right panels show that telbivudine has no effect on TNF-α (a,b) or IL-12 (c,d) in LPS-induced macrophages. Data are presented as mean ± SD for three separate experiments. *$P < 0.01$.

Telbivudine improves survival and clinical outcome *in vivo*

Telbivudine increased the survival rate in MHV-3-infected C3H mice from 18.2% to 63.6% compared with the PBS-treated controls (Fig. 3a). A composite clinical score was obtained by combining the clinical scores for all measured symptoms. Telbivudine-treated mice maintained improved composite clinical scores in all conditional categories to the end of the study (Fig. 3b).

Telbivudine reduces histological lesions in the liver

In addition to improving overall survival and clinical scores, telbivudine reduced the histological signs associated with MHV-3 infection (Fig. 3c). The livers recovered from the PBS-treated mice showed remarkable necrosis by 15 days after infection compared with the livers obtained from the uninfected control mice (the arrows in Fig. 3c indicate necrosis). In contrast, although not completely free of disease, the livers recovered from the telbivudine-treated mice showed a marked reduction in histological evidence of hepatic necrosis (Fig. 3c).

Telbivudine promotes IFN-γ production and inhibits IL-4 production *in vivo*

Telbivudine treatment resulted in an additional 1.8-fold increase in IFN-γ production in sera compared with MHV-3-infected controls (Table 1). In contrast, a 1.6-fold inhibition of IL-4 production was observed in the telbivudine-treated group compared with the controls.

Telbivudine increases the total number of T cells, the percentage of IFN-γ- and TNF-α-producing CD4$^+$ T cells, and the percentage of IFN-γ-producing CD8$^+$ T cells

Phenotypic analysis of spleen cells from telbivudine-treated mice revealed an increase in the total number of T cells compared with PBS- and lamivudine-treated controls (Fig. 4a). The frequency of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells in the telbivudine-treated group was significantly increased compared with the PBS- and lamivudine-treated groups (Figs 4b,c,f). The frequency of TNF-α-producing CD4$^+$ T cells in the telbivudine-treated group was increased significantly compared with the PBS- and lamivudine-
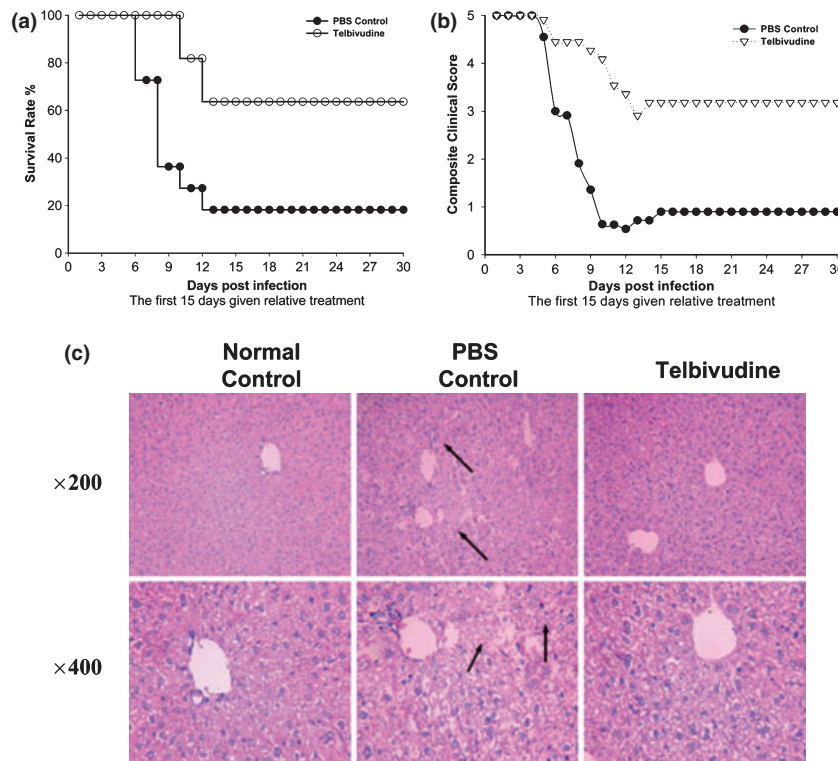

Fig. 3 Telbivudine therapy improves survival rate, clinical conditions and histological lesions in MHV-3-infected C3H mice. Fifteen animals in each group received either telbivudine or PBS for 15 days. (a) Survival rates in the telbivudine group increased to 63.6% when compared with the PBS group (18.2%). (b) Composite clinical scores: scores were calculated for fur texture, respiration and tremor. Data are the mean examination of three animals per group scored in a blinded fashion. (c) Histological improvement: at 15 days after infection, hepatic necrosis was minimized post telbivudine treatment (arrows).

Table 1 Effect of telbivudine on cytokine release following MHV-3 infection *in vivo*

| Days after MHV-3 infection in C3H mice | No infection control | | Treatment | | | |
| | | | PBS | | Telbivudine | |
	IFN-γ (ng/mL)	IL-4 (pg/mL)	IFN-γ (ng/mL)	IL-4 (pg/mL)	IFN-γ (ng/mL)	IL-4 (pg/mL)
15	17.7 ± 1.3	48.2 ± 2.0	67.4 ± 3.5	44.6 ± 2.6	125.9* ± 4.3	26.6* ± 1.8

Concentrations of IFN-γ and IL-4 were measured in sera of C3H mice 15 days after infection. Data are presented as mean ± SD from three separate experiments with three mice in each group. *$P < 0.01$ compared with PBS-treated mice.

treated groups (Figs 4d,g). The frequency of the TNF-α-producing CD8^{+} T cells was similar among all of the treatment groups (Figs 4e,g). No difference was observed in the frequency of IL-4- and IL-10-producing CD4^{+} and CD8^{+} T cells (data not shown).

Telbivudine has no effect on cytolytic function but downregulates PD-L1 expression on T cells

To determine whether telbivudine treatment enhanced killer T-cell cytotoxicity, we performed cytotoxicity assays using spleen-isolated CD8^{+} T cells as effector cells. An increase in cytotoxicity (Fig. 5a) was observed in all infected groups, but no differences were observed among groups with or without telbivudine treatment, suggesting that telbivudine has no effect on the cytolytic function of T cells. PD-1 and FasL expression were increased in infected groups compared with the uninfected group, but no differences were observed among infected groups (Fig. 5b). There were no significant differences for tumour necrosis factor receptor (TNFR)-1, TNFR-2, perforin or granzyme B expression on the CD8^{+} T cells among infected groups and the uninfected group (Fig. 5c). To further understand this phenomenon, we investigated the expression of PD-L1 and found that telbivudine treatment significantly downregulated the expression of PD-L1 on CD3^{+} and CD8^{+} T cells compared with PBS and lamivudine treatment (Figs 5d,e).

DISCUSSION

This is the first study to demonstrate, both *in vitro* and *in vivo*, that telbivudine has immunomodulatory effects, independent of its potent antiviral activity. Our data show that telbivudine has no inhibitory effects on the replication of MHV-3 in macrophages. This finding is consistent with the fact that telbivudine is highly selective for HBV and lacks activity against other viruses [12]. This study showed that telbivudine, at concentrations that are achieved *in vivo*, enhanced the production of TNF-α and IL-12 in BALB/cJ macrophages *in vitro* when challenged with MHV-3. Telbivudine was unable to cause a similar effect in LPS-induced inflammatory cytokines. This may be because the mecha-

nism of induction of TNF-α and IL-12 by LPS is different from that by virus [23].

Previous studies of the immune response during antiviral treatment with nucleoside or nucleotide analogues in patients with CHB have shown that potent suppression of HBV replication is associated with enhanced T-cell reactivity and cytokine production [24–26]. However, these studies in patients with CHB do not allow dissection of whether the effect on adaptive immunity is due to direct immunomodulatory properties of the antiviral agent or a secondary to reduced viraemia and antigen levels. The advantage of our mouse model is that it allows investigation of potential immunomodulatory effects independent of antiviral activity. The use of MHV-3, which produces a strain-dependent pattern of viral hepatitis in inbred strains of mice, has brought insights into the pathogenesis of viral hepatitis [27–31]. A previous study using an MHV-3 virus-induced hepatitis model from our group showed that ribavirin preserves Th1 cytokine production but inhibits Th2 cytokine response; the study explored the remarkable increased sustained virological response post interferon and ribavirin combination therapy for hepatitis C patients [9].

The results from the *in vitro* experiments on the MHV-3-infected macrophages support a further investigation of the immunomodulatory effect of telbivudine *in vivo*. Because MHV-3 belongs to the coronavirus family of positive-stranded RNA viruses, it is a suitable model with which to study the pure immunomodulatory effect of telbivudine without the influence of its viral inhibitory effect, which in turn may indirectly affect the immune system. In this study, telbivudine treatment significantly improved survival in MHV-3-infected C3H mice and it improved clinical conditions and histological outcomes. The treatment of MHV-3-infected C3H mice with telbivudine resulted in the inhibition of IL-4 production, and similar doses of telbivudine enhanced the production of IFN-γ, and furthermore increased total T cell numbers, IFN-γ-producing CD4^{+} and CD8^{+} T cells and TNF-α-producing CD4^{+} T cells, indicating a predominantly Th1 response post telbivudine treatment.

One mechanism of the immune response involves naive CD4^{+} T cells, which, through the production of cytokines, control the development of immune–effector mechanisms
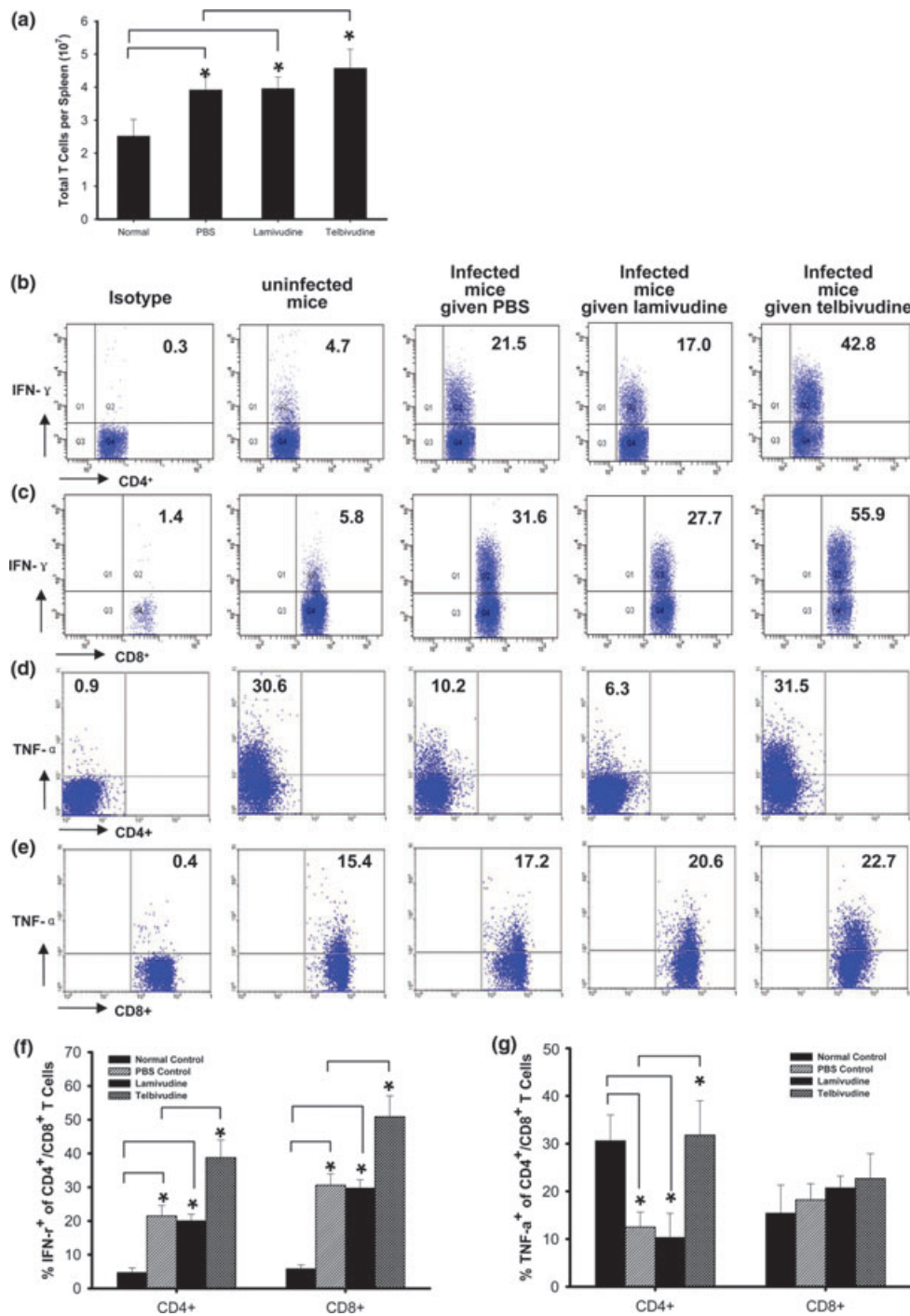
Fig. 4 Effect of telbivudine treatment on the total number of T cells and the percentage of IFN-γ and TNF-α in CD4^{+} and CD8^{+} T cells. Mice were sacrificed 15 days after infection, and single-cell suspensions were prepared from their spleen. Cells were stained with anti-CD3, anti-CD4 or anti-CD8, and IFN-γ or TNF-α. Telbivudine treatment increased the total number of T cells, which was determined by the percentage of CD3^{+} cells in counted splenocytes (a). Telbivudine increased the percentage of IFN-γ- and TNF-α-producing CD4^{+} T cells (b,d,f,g) and the percentage of IFN-γ-producing CD8^{+} T cells (c,f,g). The numbers in plots were assessed on CD4^{+} or CD8^{+} T cells. The results are a combination of three independent experiments. (b–e) are representative of three similar experiments. *$P < 0.01$.

[32]. A Th1 response has been associated with host resistance whereas a Th2 response has been associated with susceptibility in murine models of MHV-3. Studies have suggested that resistance to MHV-3 is associated with a predominant Th1 response, the production of IFN, and cytotoxic T cells [33,34]. Cytotoxic lymphocytes kill target
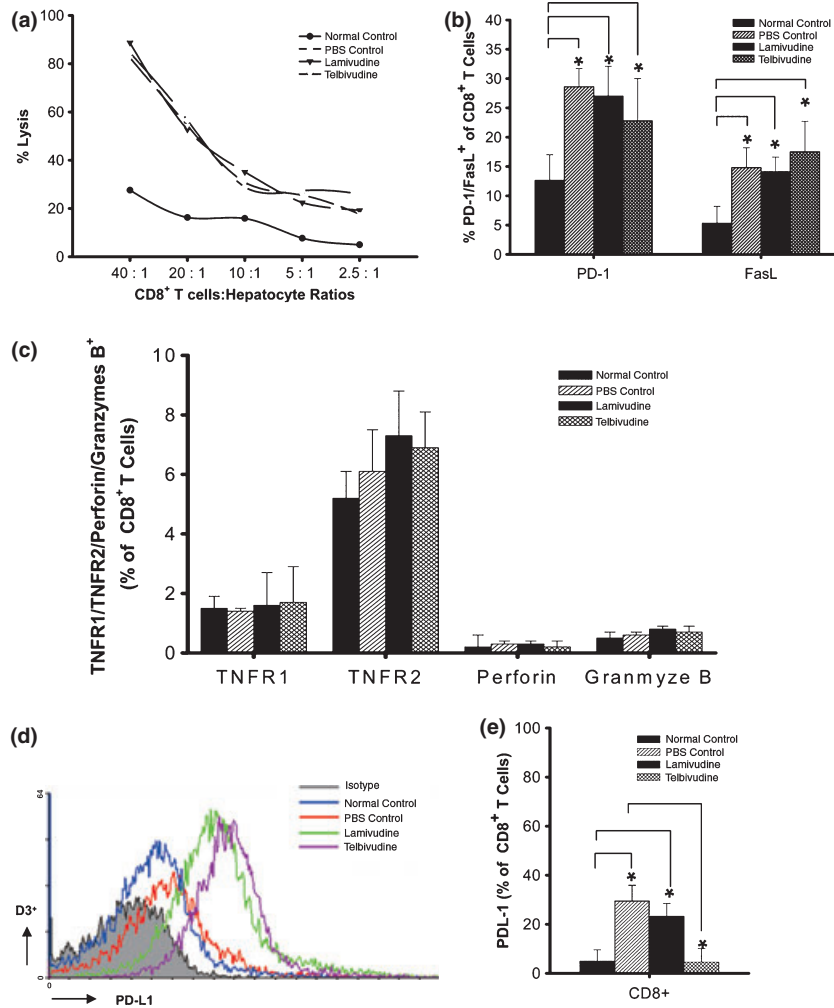
Fig. 5 Telbivudine has no effect on cytotoxicity. Hepatocytes were isolated from MHV3-infected C3H mice on Day 15 as target cells. As effector cells, CD8^{+} T cells (pooled from three mice per experimental group) were obtained from each group. (a) Cytotoxicity to hepatocytes and (b) PD-1 and Fas ligand expression on CD8^{+} T cells were increased in infected groups. There were no differences between the PBS-, lamivudine- and telbivudine-treated groups. (c) TNFR1, TNFR2, perforin and granzyme B expression on CD8^{+} T cells were similar in each group. (d) Histograms show PD-L1 expression levels on CD3^{+} cells from each group. (e) The percentage of PD-L1 expression on CD8^{+} T cells is shown. The results are a combination of three independent experiments. (d) is representative of three similar experiments. *$P < 0.01$.

cells by two major pathways: one is dependent on the exocytosis of granule effector molecules, including perforin and granzymes A and B; the other is dependent on the engagement of target cell TNFR-1 family death receptors (e.g. Fas, TNFR-1, TNFR-2) by effector cell-expressed FasL, membrane-bound or secreted TNF, or TNF-related apoptosis-inducing ligand [35–37]. Our results demonstrate that FasL plays an important role in cytotoxic T lymphocyte (CTL) cytotoxicity to hepatocytes in MHV-3-infected C3H mice. However, telbivudine treatment did not promote CTL cytotoxicity. Previous studies have established that TNF-α and IFN-γ are key cytokines for non-cytolytic control of HBV replication [38–40]. The release of these cytokines from T cells leads to inactivation of HBV without killing the infected hepatocytes. Our results suggest that telbivudine is altering

the balance of Th1/Th2 cytokines which is independent of the direct antiviral effect against MHV-3.

Programmed death-1 is a receptor that is expressed on a subset of activated T and B cells and PD-L1 (also known as B7-H1) is expressed on both haematopoietic and parenchymal cells [41,42]. In chronic viral infection, CD8 T cells express high levels of PD-1, but its ligand PD-L1 is also highly upregulated [43]. *In vivo* blockade of the interaction of PD-1 with its ligand PD-L1 has been reported to enhance T-cell responses [43]. Reduction of PD-1 during the treatment of CHB was shown to correlate with reduction of HBV DNA levels [26]. Given the role of PD-1/PD-L1 is an important inhibitory pathway in T-cell function, the effect of telbivudine on the expression of PD-1 and PD-L1 was investigated to further define the effect of telbivudine on

T-cell response. Although telbivudine does not alter the expression of PD-1, of note is that it significantly downregulated PD-L1 expression on T cells in MHV-3-infected C3H mice. This result provides initial evidence for a role of telbivudine in this inhibitory pathway in regulating T-cell function that may favour the development of immune clearance of virus infection. However, the effects of telbivudine on PD-1 and PD-L1 expression on other cell types should be clarified in further studies. Also, the underlying molecular mechanism requires further investigation. One of the proposed mechanisms is upregulation of the activity of cellular kinases to facilitate efficient phosphorylation. On the other hand, many nucleoside analogue-like substances [e.g. CpG-containing DNA, as found in DNA viruses and bacteria; viral dsRNA; the viral mimic polyinosinic:polycytidylic acid (polyI:C)] can exert immunomodulatory activities via toll-like receptors. As telbivudine is a thymidine nucleoside analogue, it may exert immunomodulatory activities via toll-like receptors.

In summary, the present study demonstrates that the beneficial effects of telbivudine in MHV-3-induced hepatitis may be mediated by its effect on the immune response rather than the inhibition of viral replication in this animal model. The immunomodulatory effect of telbivudine may be explained partly by a shift in the balance between Th1- and Th2-like immune responses. This in turn may explain the benefit of telbivudine on the amelioration of MHV-3 infection and increased survival as well as improvements in clinical scores and liver histology. These data identify an immunomodulatory mechanism of telbivudine treatment in the MHV-3-induced viral hepatitis model and provide an insight into a potential additional mode of action for the management of viral hepatitis infection.

ACKNOWLEDGEMENTS

STATEMENT OF INTERESTS

REFERENCES

1 World Health Organization. Hepatitis B Fact Sheet. Available at: http://www.who.int/mediacentre/factsheets/fs204/en/ (accessed 31 January 2009).

2 Ferrari C, Penna A, Bertoletti A *et al.* Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J Immunol* 1990; 145: 3442–3449.

3 Rehermann B, Fowler P, Sidney J *et al.* The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J Exp Med* 1995; 181: 1047–1058.

4 Thimme R, Wieland S, Steiger C *et al.* CD8 (+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 2003; 77: 68–76.

5 Penna A, Artini M, Cavalli A *et al.* Long-lasting memory T cell responses following self-limited acute hepatitis B. *J Clin Invest* 1996; 98: 1185–1194.

6 European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B. *J Hepatol* 2009; 50: 227–242.

7 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–539.

8 Tilg H. New insights into the mechanisms of interferon alfa: an immunoregulatory and anti-inflammatory cytokine. *Gastroenterology* 1997; 112: 1017–1021.

9 Ning Q, Brown D, Parodo J *et al.* Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. *J Immunol* 1998; 160: 3487–3493.

10 Ning Q, Lakatoo S, Liu M *et al.* Induction of prothrombinase fgl2 by the nucleocapsid protein of virulent mouse hepatitis virus is dependent on host hepatic nuclear factor-4 alpha. *J Biol Chem* 2003; 278: 15541–15549.

11 Liu WL, Su WC, Cheng CW *et al.* Ribavirin up-regulates the activity of double-stranded RNA-activated protein kinase and enhances the action of interferon-alpha against hepatitis C virus. *J Infect Dis* 2007; 196: 425–434.

12 Bryant ML, Bridges EG, Placidi L *et al.* Antiviral L-nucleosides specific for hepatitis B virus infection. *Antimicrob Agents Chemother* 2001; 45: 229–235.

13 Golitsina N, Danehy F, Fellows R. Telbivudine phosphorylation by three enzymes: implications for antihepatitis B virus activity in vitro and in the clinic. *Hepatology* 2006; 44: 561A.

14 Hernandez-Santiago B, Placidi L, Cretton-Scott E *et al.* Pharmacology of beta-L-thymidine and beta-L-2′-deoxycytidine in HepG2 cells and primary human hepatocytes: relevance to chemotherapeutic efficacy against hepatitis B virus. *Antimicrob Agents Chemother* 2002; 46: 1728–1733.

15 Seifer M, Patty A, Dukhan D. Telbivudine (LDT) preferentially inhibits second (+) strand HBV DNA synthesis. *J Hepatol* 2005; 42: 151.

16 Lai CL, Gane E, Liaw YF *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; 357: 2576–2588.

17 Liaw YF, Gane E, Leung N *et al.* 2-Year GLOBE trial results: telbivudine is superior to lamivudine in patients with chronic hepatitis B. *Gastroenterology* 2009; 136: 389–392.

18 Qin XM, Zhang JG, Su K, Yang C, Yan WM, Ning Q. Establishment and characterization of a chronic viral hepatitis model induced by murine hepatitis virus type 3. *Virol Sin* 2006; 21: 417–420.

19 Zhang J, Qin X, Wang XJ *et al.* A primary study of the subgroups of T lymphocytes in MHV-3 induced chronic viral hepatitis. *Virol Sin* 2007; 22: 339–346.

20 Levy GA, Adamson G, Phillips MJ *et al.* Targeted delivery of ribavirin improves outcome of murine viral fulminant hepatitis via enhanced anti-viral activity. *Hepatology* 2006; 43: 581–591.

21 Hu P, Jiang J, Wang H *et al.* Single-dose and multiple-dose pharmacokinetics and safety of telbivudine after oral administration in healthy Chinese subjects. *J Clin Pharmacol* 2006; 46: 999–1007.

22 Zhou XJ, Lim SG, Lloyd DM, Chao GC, Brown NA, Lai CL. Pharmacokinetics of telbivudine following oral administration of escalating single and multiple doses in patients with chronic hepatitis B virus infection: pharmacodynamic implications. *Antimicrob Agents Chemother* 2006; 50: 874–879.

23 Shigemi S, Tsutsumi H, Takeuchi R, Matsuda K, Imai S, Ogra PL. Enhanced cytokine production by milk macrophages following infection with respiratory syncytial virus. *J Leukoc Biol* 1997; 61: 630–636.

24 Boni C, Penna A, Bertoletti A *et al.* Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. *J Hepatol* 2003; 39: 595–605.

25 Cooksley H, Chokshi S, Maayan Y *et al.* Hepatitis B virus e antigen loss during adefovir dipivoxil therapy is associated with enhanced virus-specific CD4$^+$ T-cell reactivity. *Antimicrob Agents Chemother* 2008; 52: 312–320.

26 Evans A, Riva A, Cooksley H *et al.* Programmed death 1 expression during antiviral treatment of chronic hepatitis B: impact of hepatitis B e-antigen seroconversion. *Hepatology* 2008; 48: 759–769.

27 Ding JW, Ning Q, Liu MF *et al.* Fulminant hepatic failure in murine hepatitis virus strain 3 infection: tissue-specific expression of a novel fgl2 prothrombinase. *J Virol* 1997; 71: 9223–9230.

28 Ding JW, Ning Q, Liu MF *et al.* Expression of the fgl2 and its protein product (prothrombinase) in tissues during murine hepatitis virus strain-3 (MHV-3) infection. *Adv Exp Med Biol* 1998; 440: 609–618.

29 Levy GA, Liu M, Ding J *et al.* Molecular and functional analysis of the human prothrombinase gene (HFGL2) and its role in viral hepatitis. *Am J Pathol* 2000; 156: 1217–1225.

30 Marsden PA, Ning Q, Fung LS *et al.* The Fgl2/fibroleukin prothrombinase contributes to immunologically mediated thrombosis in experimental and human viral hepatitis. *J Clin Invest* 2003; 112: 58–66.

31 Zhu C, Sun Y, Luo X, Yan W, Xi D, Ning Q. Novel mfgl2 antisense plasmid inhibits murine fgl2 expression and ameliorates murine hepatitis virus type 3-induced fulminant hepatitis in BALB/cJ mice. *Hum Gene Ther* 2006; 17: 589–600.

32 Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7: 145–173.

33 Levy GA, Shaw R, Leibowitz JL, Cole E. The immune response to mouse hepatitis virus: genetic variation in antibody response and disease. *Adv Exp Med Biol* 1984; 173: 345–364.

34 Pope M, Chung SW, Mosmann T, Leibowitz JL, Gorczynski RM, Levy GA. Resistance of naive mice to murine hepatitis virus strain 3 (MHV-3) requires development of Th1, but not Th2 response, whereas pre-existing antibody protects against primary infection. *J Immunol* 1996; 156: 3342–3349.

35 Kägi D, Vignaux F, Ledermann B *et al.* Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 1994; 265: 528–530.

36 Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus host disease. *J Exp Med* 1996; 183: 657–661.

37 Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* 2002; 20: 323–370.

38 Chisari FV. Hepatitis B virus transgenic mice: insights into the virus and the disease. *Hepatology* 1995; 22: 1316–1325.

39 Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* 2001; 19: 65–91.

40 Suri D, Schilling R, Lopes AR *et al.* Non-cytolytic inhibition of hepatitis B virus replication in human hepatocytes. *J Hepatol* 2001; 35: 790–797.

41 Agata Y, Kawasaki A, Nishimura H *et al.* Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996; 8: 765–772.

42 Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol* 2001; 22: 265–268.

43 Barber DL, Wherry EJ, Masopust D *et al.* Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; 439: 682–687.