

# Thymosin $\alpha 1$ accelerates restoration of T cell-mediated neutralizing antibody response in immunocompromised hosts

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## Abstract

Thymosin  $\alpha 1$  is a biological response modifier that has been used clinically for the treatment of chronic hepatitis B viral infection. Both immunomodulatory and immediate intracellular mechanisms have been postulated to explain the effect of thymosin  $\alpha 1$  on hepatocytes infected with hepatitis B virus (HBV). Here, we established a new animal model and the related suitable conditions to access the thymosin activity by means of measuring the production of neutralizing antibody against hepatitis B surface antigen (HBsAg). We proved that chemically synthesized thymosin  $\alpha 1$  restored the T cell-mediated antibody production following its suppression in mice by 5-fluorouracil (5-FU), and found that thymosin  $\alpha 1$  showed activity at a low dose of 30  $\mu\text{g}/\text{kg}$ . Further studies utilizing the flowcytometric analysis showed that thymosin  $\alpha 1$  at this dose accelerated the replenishment and maturation of thymocytes while the expression of Smoothed (Smo) of the Hedgehog (Hh)-signaling in  $\text{CD4}^- \text{CD8}^-$  thymocytes, the potent negative regulator of proliferative responses, was not affected. The restoration of some of the defects in the host defense systems may facilitate elimination of infectious agents, and the present study provides a novel model to define the restoration of T cell-mediated immune responses to hepatitis B virus in vivo. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Hepatitis B virus; Thymosin; Thymus; 5-FU; Hedgehog

## 1. Introduction

The thymus is an endocrine gland indispensable for the development and subsequent maturation of thymocytes. A number of studies concerning thymic functions led to the discovery of various hormonal-like factors such as thymosin, serum thymic factor

and thymopoietin [1–4]. The activities of these factors have been evaluated in immunological assay systems in vivo, ex vivo, and in vitro as reviewed by Bach [5]. Thymosin fraction 5 [1], a partially purified preparation from calf thymus, has been studied most extensively in clinical trials. It was proven to be quite effective in patients with some immunodeficiency diseases and in patients with either chronic hepatitis B virus (HBV) infection [6–10] or with chronic hepatitis C infection [10–14].

HBV is a noncytopathic virus and the persistence of HBV infection is thought to be due primarily to a deficient host immune response [2]. Patients

*Abbreviations:* 5-FU, 5-fluorouracil; anti-HBs, anti-hepatitis B virus surface antigen; HBV, hepatitis B virus; Hh, Hedgehog; Smo, Smoothed.

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with chronic hepatitis B have a large amount of HBV-specific CD8<sup>+</sup> cells, but may be deficient in HBV-specific CD4<sup>+</sup> T cells [15,16]. During the establishment of viral persistence, the rapid loss of HBV-specific CD4<sup>+</sup> T cell responsiveness may play a key role in the failure of the infected host to mount effective neutralizing antibody responses against HBV. HBV-specific CD4<sup>+</sup> cells may be involved in controlling viral replication, supporting the expansion of HBV-specific CD8<sup>+</sup> cells that involved in eliminating HBV-infected hepatocytes [15], and producing neutralizing antibody. The loss of specific CD4<sup>+</sup> T cells may cause a decrease in the production of neutralizing antibodies against HBV, and affect the long-term immune control of HBV. The recognition of infected hepatocytes by HBV-specific T cells has been assumed to be the central mechanism causing both liver damage and viral control [15,16].

The mammalian Hedgehog (Hh) is a family of secreted proteins, which includes sonic Hh, Indian Hh and desert Hh, plays major role in many patterning processes during animal development. Hh proteins signal to nearby cells through two transmembrane proteins—Patched (Ptc) and Smoothed (Smo). Smo is the co-receptor and the transducer of Hh signal [17]. Smo is stage-specifically expressed in CD4<sup>-</sup>CD8<sup>-</sup> double-negative thymocytes, and its downregulation is necessary for the progression of this population to CD4<sup>+</sup>CD8<sup>+</sup> double-positive stage; therefore, Hh-signaling is an important regulatory pathway of intrathymic T cell maturation [18].

Either 5-fluorouracil (5-FU) or cyclophosphamide (CY) treatment is classical model for immunosuppression to establish the immunocompromised state [19]. Here, we developed a simple *in vivo* model for measuring the HBV-specific immune response in immunocompromised hosts and have shown in the present work that this model can be used in accessing the immunomodulating activity of thymosin  $\alpha$ 1 *in vivo*.

## 2. Experimental procedures

### 2.1. Animals and reagents

Female C3H/He mice of 7 weeks of age were purchased from Shizuoka Agricultural Cooperative

Association for Laboratory Animals (Hamamatsu, Japan). Thymosin  $\alpha$ 1, a synthetic polypeptide of thymic origin (Alpha 1, Biomedicals, WA) [20], and 5-FU (Kyowa Hakkō, Tokyo) were dissolved in physiological saline for use.

Mice were injected intraperitoneally with 0.2 ml of physiological saline containing 25 mg/kg of 5-FU and various concentrations of thymosin  $\alpha$ 1 (0, 0.3, 3, 30  $\mu$ g/kg) for 10 consecutive days. Mice treated with 0.2 ml of physiological saline were used as the control group.

Twenty-four hours after the last administration of 5-FU, 21 mice of each group were divided into two groups: 15 mice were immunized intraperitoneally with 4 mg/mouse of recombinant hepatitis B surface antigen (HBsAg, Heptavax, Merck). Thymi of the other six mice were used for weighing and flowcytometric analysis.

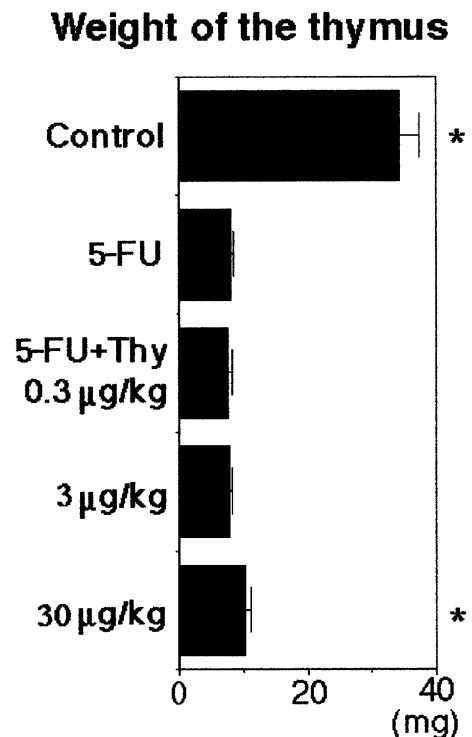


Fig. 1. Effects of 5-FU and thymosin  $\alpha$ 1 on thymus weight. 5-FU treatment induced serious thymic atrophy, but co-administration of thymosin  $\alpha$ 1 30  $\mu$ g/kg attenuated the change significantly ( $P < 0.01$ ).

The sera of five mice in each group were collected for measuring the titer of anti-HBs at 10, 15 and 20 days after the immunization.

## 2.2. Flowcytometry analysis

One million freshly isolated thymocytes were stained with the antibodies, CD4 (clone GK1.5, PharMingen, San Diego, CA), CD8 (clone KT15, Immunotec). One million cells of CD4<sup>-</sup>CD8<sup>-</sup> thymocytes prepared by negative selection [21] were stained with goat anti-mouse Smo (N-19) (Santa Cruz Biotechnology). Cells were analyzed using a FACScan (Becton Dickinson, San Jose, CA) and CellQuest software.

## 2.3. Quantification of anti-HBs in mice sera

One hundred microliters of sequentially diluted positive control sera (413 IU/l) or sample sera were put into wells of 98-well plates coated with HBsAg (Alpha Therapeutic, Los Angeles, CA) in triplicate. Plates were incubated for 2 h at room temperature. After quenching, wells were filled with 100  $\mu$ l of biotinylated goat anti-mouse IgG (MBL, Japan) and incubated for 1 h at room temperature. After quenching, wells were filled with 100  $\mu$ l of avidin–biotinylated peroxidase complex for 20 min at room temperature. After quenching, wells were filled with 100  $\mu$ l of substrate solution (R&D Systems, Minneapolis, MN). One hundred microliters of stop solu-

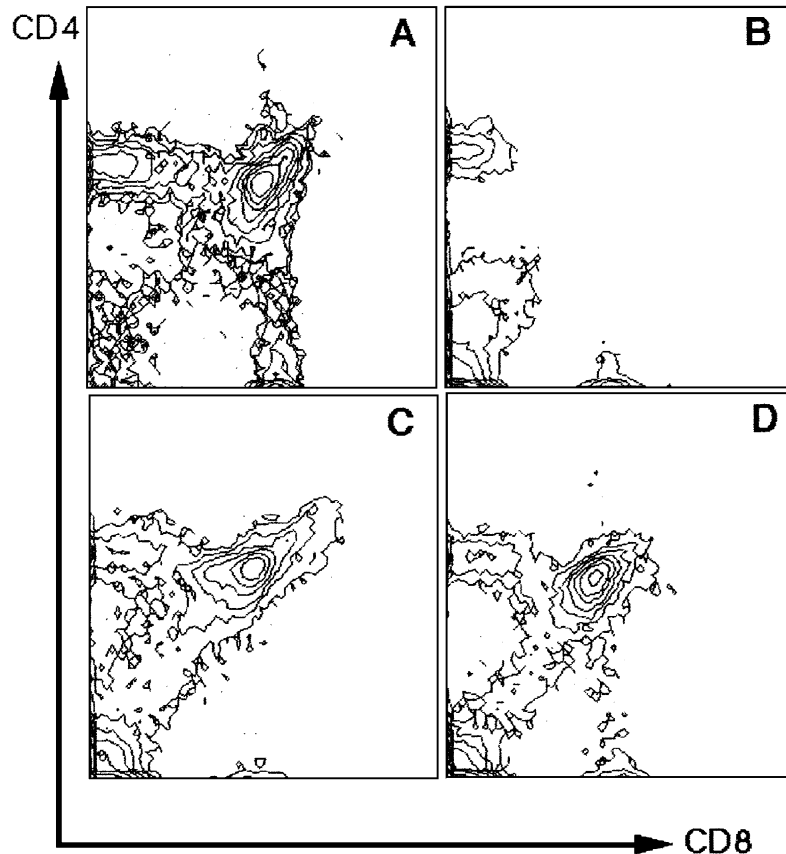


Fig. 2. Flowcytometrical analysis of thymocyte subpopulation. 5-FU treatment eliminated CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes. Co-administration of thymosin  $\alpha$ 1 accelerated the restoration of CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocyte population. (A) Control mouse; (B) mouse treated with 5-FU for 10 days; (C) mouse treated with 5-FU for 10 consecutive days and rested for 10 days; (D) mouse treated with 5-FU + thymosin  $\alpha$ 1 30  $\mu$ g/kg for 10 consecutive days and rested for 10 days.

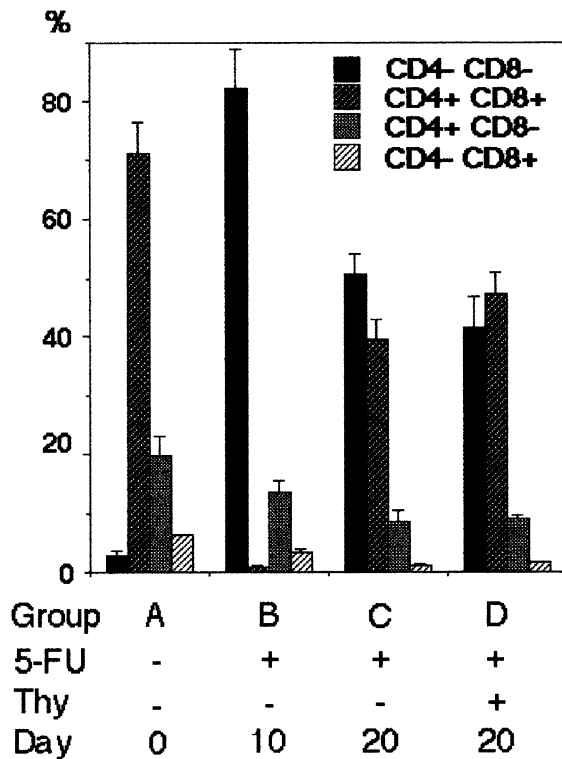


Fig. 3. Sequential analysis of the effects of 5-FU and thymosin  $\alpha 1$  on thymocyte phenotypes. 5-FU treatment suppressed the progression of  $CD4^- CD8^-$  double-negative thymocytes to  $CD4^+ CD8^+$  double-positive thymocytes. Co-administration of thymosin  $\alpha 1$  with 5-FU accelerated the restoration of  $CD4^+ CD8^+$  double-positive thymocyte population. (A) Control mouse; (B) mouse treated with 5-FU for 10 days; analysis was done on day 10; (C) mouse treated with 5-FU for 10 consecutive days and rested for 10 days; analysis was done on day 20; (D) mouse treated with 5-FU+thymosin  $\alpha 1$  30  $\mu\text{g}/\text{kg}$  for 10 consecutive days and rested for 10 days; analysis was done on day 20.

tion was added to determine optical density of each well using a microplate reader set to 450 nm (Multiskan Plus MKII, Flow Laboratories Japan, Tokyo).

#### 2.4. Statistical analysis

Group means and standard deviation (S.D.) were calculated using Excel Statistics (ver. 4.0, Microsoft). Dose–response studies were evaluated by ANOVA and  $P < 0.05$  was considered statistically significant using Student's *t*-test and Dunnett's multiple comparison test.

### 3. Results

#### 3.1. Evaluation of the thymosin activity by the weight of thymus

Intraperitoneal administration of 25 mg/kg of 5-FU for 10 days was sufficient to induce immune suppression in mice because there was a significant decrease in the weight of the thymus ( $P < 0.001$ ): the weight of the thymus was  $34.4 \pm 3.0$  mg (mean

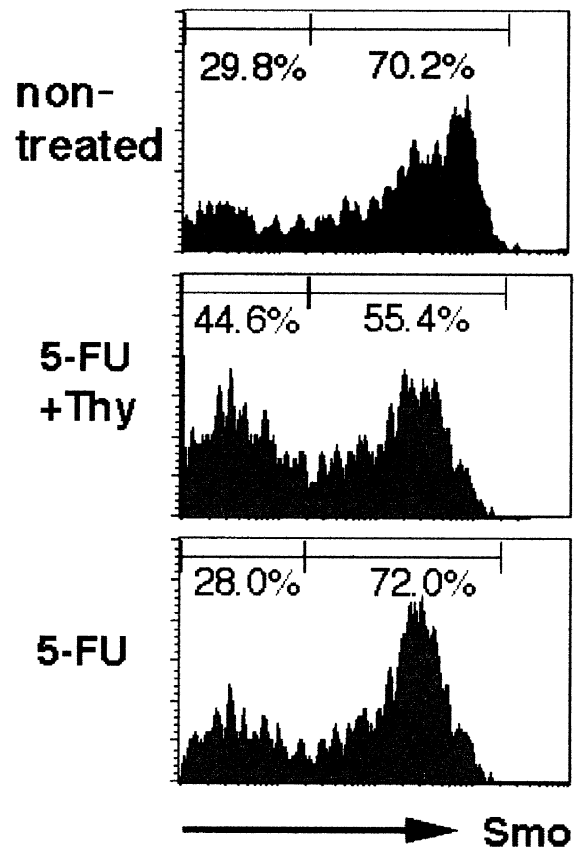


Fig. 4. Effects of 5-FU and thymosin  $\alpha 1$  on expression of Smo in  $CD4^- CD8^-$  double-negative thymocyte population. Smo is stage-specifically expressed in  $CD4^- CD8^-$  double-negative thymocytes, and its downregulation is necessary for the progression of this population to  $CD4^+ CD8^+$  double-positive stage; 5-FU treatment did not affect the frequency of Smo $^-$  population. Thymosin  $\alpha 1$  accelerated the accumulation of Smo $^-$  thymocytes ready for the progression to  $CD4^+ CD8^+$  double-positive thymocytes.

$\pm$  S.D.) in the control mice and  $8.0 \pm 0.2$  mg in mice treated with 5-FU. The weight of the thymus was restored with co-administration of thymosin  $\alpha 1$  (Fig. 1): the weight of the thymus was  $7.7 \pm 0.3$  mg in mice treated with  $0.3 \mu\text{g}/\text{kg}$  of thymosin  $\alpha 1$ ,  $7.8 \pm 1.9$  mg in mice treated with  $3 \mu\text{g}/\text{kg}$  of thymosin  $\alpha 1$  and  $10.3 \pm 0.8$  mg in mice treated with  $30 \mu\text{g}/\text{kg}$  of thymosin  $\alpha 1$ . Co-administration of  $30 \mu\text{g}/\text{kg}$  of thymosin  $\alpha 1$  significantly reverted the weight of the thymus (Dunnett's multiple comparison test,  $P < 0.01$ ).

### 3.2. Evaluation of the thymosin activity by the flow-cytometry

Results of a typical experiment are shown in Fig. 2, and the results are summarized in Fig. 3. Intraperitoneal administration of  $25 \text{ mg}/\text{kg}$  of 5-FU for 10

days was sufficient to induce immune suppression in mice because there was a significant decrease in the proportion of  $\text{CD4}^+\text{CD8}^+$  double-positive cells in the thymus ( $77.1 \pm 5.3\%$  vs.  $0.8 \pm 0.4\%$ ,  $P < 0.001$ , Fig. 3). The major population of thymocytes in the control mice (Fig. 2A) were  $\text{CD4}^+\text{CD8}^+$  double-positive cells which accounts for  $77.1 \pm 5.3\%$  of total thymocytes, whereas that in 5-FU-treated mice were  $\text{CD4}^-\text{CD8}^+$  double-negative cells (Fig. 2B) which accounts for  $82.2 \pm 4.9\%$  of total thymocytes (Fig. 2B). The dominant population has become  $\text{CD4}^+\text{CD8}^+$  double-positive cells at 10 days after the immunization in mice co-administrated with  $30 \mu\text{g}/\text{kg}$  of thymosin  $\alpha 1$  ( $47.4 \pm 3.7\%$ ) (Figs. 2D and 3), whereas  $\text{CD4}^-\text{CD8}^+$  double-negative cells were the dominant population in mice treated with 5-FU without thymosin  $\alpha 1$  ( $50.7 \pm 3.3\%$ ) (Figs. 2C and 3).

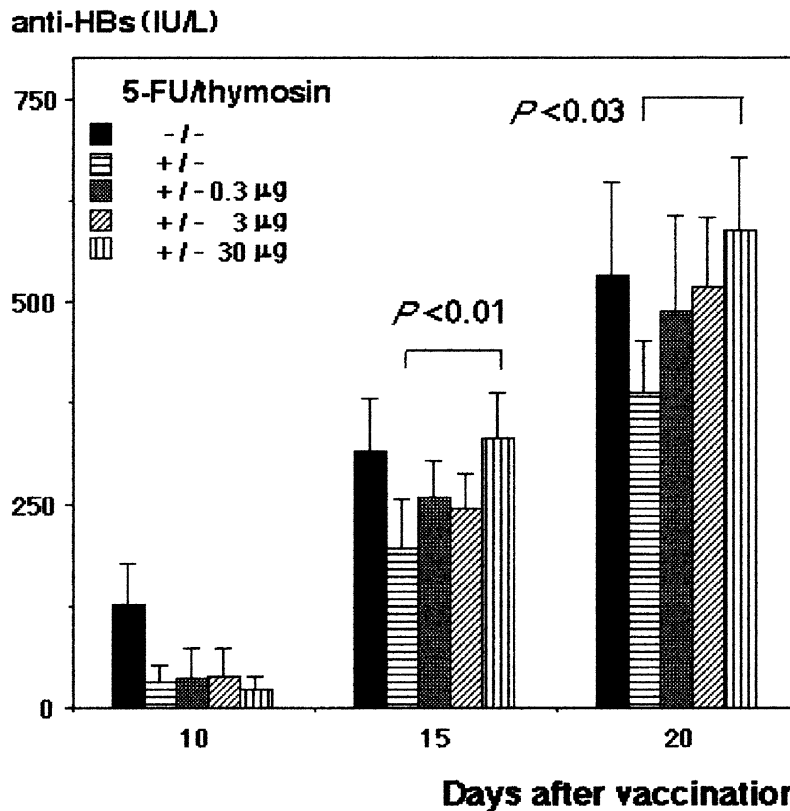


Fig. 5. Effects of 5-FU and thymosin  $\alpha 1$  on anti-HBs titer. 5-FU treatment suppressed immune response to HBs, but co-administration of thymosin  $\alpha 1$   $30 \mu\text{g}/\text{kg}$  attenuated the change significantly ( $P < 0.01$  on day 15 and  $P < 0.03$  on day 20 after vaccination,  $n = 5$ ). Note that days 10, 15, and 20 after vaccination correspond to days 20, 25, and 30 after the start of 5-FU treatment.

### 3.3. Effects of 5-FU and thymosin on expression of Smo of the Hh-signaling in CD4<sup>-</sup>CD8<sup>-</sup> double-negative thymocyte population

Smo expression in CD4<sup>-</sup>CD8<sup>-</sup> double-negative thymocyte population was assessed in mice treated with 5-FU for 10 consecutive days and mice treated with 5-FU + thymosin  $\alpha$ 1 30  $\mu$ g/kg for 10 consecutive days (Fig. 4). The peak intensity of Smo expressed in Smo<sup>+</sup> population of 5-FU-treated mice was  $277.0 \pm 13.8$ , and this value was similar to those observed in control mice ( $263.4 \pm 15.1$ ) and in thymosin co-administered mice ( $259.4 \pm 11.7$ ). The proportion of Smo<sup>-</sup> thymocytes in 5-FU-treated mice was  $26.9 \pm 4.1\%$ , and this value was similar to the value  $28.6 \pm 4.5\%$  observed in control mice, whereas it was significantly larger in mice co-administered with thymosin  $\alpha$ 1 ( $44.1 \pm 5.2\%$ ,  $P < 0.001$ ).

### 3.4. Evaluation of the thymosin activity by measuring the titer of anti-HBV surface antigen (anti-HBs)

Intraperitoneal administration of 25 mg/kg of 5-FU for 10 consecutive days was sufficient to induce immune suppression in mice because there was a significant decrease in the titer of anti-HBs at 10, 15 and 20 days after the immunization ( $P < 0.003$ , 0.01 and 0.02, respectively): the titers of anti-HBs were  $169.0 \pm 53.8$ ,  $315.8 \pm 65.1$  and  $532.3 \pm 116.1$  IU/l (mean  $\pm$  S.D.) in the control mice, and  $59.7 \pm 39.2$ ,  $195.5 \pm 60.2$  and  $388.0 \pm 63.1$  IU/l in mice treated with 5-FU at 10, 15 and 20 days after the immunization, respectively (Fig. 5). The titer of anti-HBs at 15 and 20 days after the immunization was restored by co-administration of 30  $\mu$ g/kg of thymosin  $\alpha$ 1 (Dunnett's multiple comparison test,  $P < 0.01$  and  $P < 0.03$ , respectively): the titers of anti-HBs were  $332.1 \pm 54.8$  and  $588.6 \pm 88.5$  IU/l at 15 and 20 days after the immunization in mice treated with 30  $\mu$ g/kg of thymosin  $\alpha$ 1, respectively.

## 4. Discussion

5-FU causes severe damage to the thymocyte populations as shown in Fig. 1. Flowcytometric analysis revealed that the most sensitive subpopulation to 5-FU was the CD4<sup>+</sup>CD8<sup>+</sup> double-positive cell since

the proportion of CD4<sup>+</sup>CD8<sup>+</sup> double-positive cells, proliferating and progressing to CD4<sup>+</sup> or CD8<sup>+</sup> single-positive mature thymocytes, were profoundly reduced in mice treated with 5-FU. Intraperitoneal administration of 30  $\mu$ g/kg of thymosin  $\alpha$ 1, which is in the range of clinical and experimental usage [6–14,19], accelerated restoration of the proportion of the CD4<sup>+</sup>CD8<sup>+</sup> double-positive cell population (Figs. 2 and 3). Therefore, thymosin  $\alpha$ 1 restored the defective immune response to HBV in these immunocompromised mice.

The restoration of the proportion of CD4<sup>+</sup>CD8<sup>+</sup> double-positive cells in the thymus may be mainly due to accelerated progression of CD4<sup>-</sup>CD8<sup>-</sup> double-negative cells to CD4<sup>+</sup>CD8<sup>+</sup> double-positive stage, but not due to the prevention of apoptosis in CD4<sup>+</sup>CD8<sup>+</sup> cell population, because 10–100-fold more amount of thymosin  $\alpha$ 1 is needed to prevent apoptosis of these cells in vitro [22]. The membrane co-receptor Smo is the signal transducer of Hh-signaling and the only molecule that negatively regulates the progression of CD4<sup>-</sup>CD8<sup>-</sup> double-negative cells to the CD4<sup>+</sup>CD8<sup>+</sup> stage. Thymocytes pass through a series of stages during T cell development: CD4<sup>-</sup>CD8<sup>-</sup> double-negative thymocytes progress to the CD4<sup>+</sup>CD8<sup>+</sup> double-positive cells and then become mature either CD4<sup>+</sup> or CD8<sup>+</sup> single-positive T cells. The double-negative population is further subdivided into four subsets: CD44<sup>+</sup>CD25<sup>-</sup> cells acquire CD25 expression (CD44<sup>+</sup>CD25<sup>+</sup>), lose CD44 expression (CD44<sup>-</sup>CD25<sup>+</sup>) in the process of T cell receptor  $\beta$  (TCR $\beta$ ) rearrangement, and finally become CD44<sup>-</sup>CD25<sup>-</sup> cells which further differentiate to CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes [23]. Three important progressions take place during the CD25<sup>+</sup> double-negative stage: (i) the TCR $\beta$  gene rearrangement takes place when CD44<sup>+</sup>CD25<sup>+</sup> cells progress to CD44<sup>-</sup>CD25<sup>+</sup>, (ii) CD44<sup>-</sup>CD25<sup>+</sup> cells express pre-TCR after a successful TCR $\beta$  gene rearrangement, (iii) a pre-TCR signaling abruptly down-regulates Smo expression during the differentiation of CD44<sup>-</sup>CD25<sup>+</sup> cells to CD44<sup>-</sup>CD25<sup>-</sup> cells and allows CD44<sup>-</sup>CD25<sup>-</sup> cells to differentiate to CD4<sup>+</sup>CD8<sup>+</sup> double-positive cells [18]. Thymocyte repertoire may be dramatically changed by the modification of Hh-signaling. Our results show that neither 5-FU nor thymosin  $\alpha$ 1 changed Smo expression level in differentiating CD4<sup>-</sup>CD8<sup>-</sup> double-negative

thymocytes, but that thymosin  $\alpha 1$  facilitated the accumulation of Smo<sup>-</sup> thymocytes ready for the progression to CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes. Therefore, the accelerated progression was possibly a direct and/or indirect effect of thymosin  $\alpha 1$  since a number of thymic factors other than thymosin  $\alpha 1$ , e.g. thymulin, thymopoietin, IL-7 and IL-1, are capable of supporting the entry and growth of T cell progenitors, and their corresponding signaling pathways may also function in this model [23]. If thymosin  $\alpha 1$  is acting in the context of other cytokines, a more careful evaluation with respect to comparing in vitro results to in vivo studies is needed.

Corresponding to the restoration of thymocyte populations in 5-FU-treated mice, thymosin  $\alpha 1$  also restored the production of anti-HBs. The activity of thymosin  $\alpha 1$  was evident when 30  $\mu\text{g}/\text{kg}$  of thymosin  $\alpha 1$  was administered intraperitoneally. This condition is suitable for assessing the thymosin  $\alpha 1$  activity in vivo in such immunocompromised hosts by measuring the humoral neutralizing antibody response to HBs. Interestingly, humoral neutralizing antibody response to HBV is T cell-dependent [24]. Presumably, the restoration of neutralizing antibody is related to the restoration of CD4<sup>+</sup> T cells. Thymosin  $\alpha 1$  was reported to have significant potentiating effects on thymocyte maturation as shown in Figs. 2 and 3 and immunologic functions which include promotion of IFN- $\gamma$ , interleukin-2 and interleukin-2 receptor production, enhanced proliferation of T cells in response to mitogenic and antigen stimulation, increased antibody synthesis and modulation of T cell cytotoxicity and helper function [5,6]. There have been some reports that thymosin is effective in regeneration of T cells or lymphoid tissue during radiation therapy [25]. These and other enhancing attributes in the immune system had led to the use thymosin  $\alpha 1$  for the treatment of chronic hepatitis B patients [5,6,26].

The present study confirmed that thymosin  $\alpha 1$  accelerated restoration of the proportion of CD4<sup>+</sup>CD8<sup>+</sup> double-positive cell population in the thymus of 5-FU-treated mice and provided an evidence for the first time that this acceleration may contribute to the restoration of T cell-mediated immune response to HBV. This observation is in agreement with a previous observation that thymosin  $\alpha 1$  was suggested to restore the function (or number) of

the mature T cells transferring the delayed immune reaction to the recipient mice [27]. Therefore, the restoration of some of the defects in the host defense systems may prevent microbial infections and may facilitate HBV elimination, and the present study provides a novel model to define restoration of T cell-mediated immune responses specific to HBV in vivo.

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