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Acute Exacerbations of Chronic Hepatitis B Are Accompanied by Decline of Core Antigen-Specific Regulatory T-Cell Frequencies: Implications for Successful Anti-HBV Treatments

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Background and Aims: **Acute exacerbations (AEs) of perinatally-acquired chronic hepatitis B (CHB) are accompanied by increased T cell responses to hepatitis B core and e antigens (HBcAg & HBeAg). Naturally-arising forkhead transcription factor Foxp3 (forkhead box p3)-expressing $CD4^+CD25^+$ regulatory T (T_{reg}) cells are thought to be important in the control of infectious diseases. This study aimed to investigate whether HBcAg-specific T_{reg} cells play a role in modulating spontaneous AEs and in influencing the outcome of anti-hepatitis B virus (HBV) treatments.**

Methods: **The SYFPEITHI scoring system was employed to predict epitope peptides on HBcAg overlapping with HBeAg for the construction of peptide-HLA class II tetramers to measure HBcAg-specific T_{reg} cell frequencies (T_{reg} *f*).**

Results: **HBcAg-specific T_{reg} *f* declined significantly in association with increased HBcAg-specific cytotoxic T lymphocyte frequencies during spontaneous AEs without treatment. Vigorous *in vitro* expansion of $CD4^+CD25^+$ T_{reg} cells from CHB patients responding to HBcAg and/or its peptides plus interleukin-2 (IL-2) was consistently detected. Depletion of T_{reg} cells from peripheral blood mononuclear cells enhanced proliferation to HBcAg. In contrast, patients with AEs who received anti-HBV treatments with oral nucleoside analogues or interferon-alpha injection revealed that more post-treatment increase of HBcAg-specific T_{reg} *f* correlated with a higher sustained remission rate to the therapy.**

Conclusions: **These data indicate that HBcAg-specific T_{reg} cells from perinatally-acquired CHB patients are proliferative to HBcAg and its peptides and exhibit suppressor activity. They play a crucial role in modulating spontaneous AEs and in successful anti-HBV treatments.**

Keywords: **Chronic Hepatitis B, Acute Exacerbation, Regulatory T Cell, Sustained Remission**

Introduction

Hepatitis B virus (HBV) infects more than 350 million people worldwide. All the infected subjects are at risk of developing adverse *sequelae* including acute fulminant hepatitis and active chronic hepatitis B (CHB) with severe acute exacerbations (AEs) or flares that may lead to acute liver failure and mortality. Long-term chronic HBV infection may progress to liver cirrhosis and hepatocellular carcinoma (HCC) [1-7]. Since the pilot clinical trials of interferon-alpha (IFN-α) therapy for CHB patients reported in 1976 [8, 9], the treatment of CHB has exhausted tremendous medical expenditure worldwide in past 30 years [6, 10, 11].

Disappointingly, CHB patients have thus far responded with low sustained remission (SR) rate to all currently available anti-HBV treatment modalities including oral nucleoside analogues such

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as lamivudine (3TC), adefovir dipivoxil (ADV), entecavir (ETV) and telbivudine (Ldt) as well as IFN-α or thymosin-α injection [6, 8-22]. Immediate anti-HBV treatment with nucleoside analogues for CHB patients with severe flare-up has achieved a great success in rescuing a large proportion of patients from hepatic failure and mortality [6, 13, 17-19, 21]. However, nucleoside analogues can only suppress virus replication via inhibiting HBV-DNA polymerase but are unable to induce a total loss of covalently closed circular (ccc) DNA of HBV genome to achieve a complete viral clearance [7, 23, 24].

Consequently, virological and/or biochemical relapses are frequently encountered when these drugs are ceased to use. Moreover, long term use of nucleoside analogues may induce the appearance of drug-resistant mutant viruses such as mutations on the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV-DNA polymerase which most frequently appear in patients receiving lamivudine therapy, resulting in treatment failure [6, 21, 22, 25]. Gaining insight into the immunopathogenesis of AEs on the natural history of CHB may facilitate in better treatment, and ultimately in preventing the disease progression [3-7, 13, 21].

The HBV is not directly cytopathic, and the immune response of the host appears to mediate the hepatocellular injury and subsequent viral clearance [3-7, 26]. Accumulating evidence indicates that the immune response of HBV patients may influence the outcome of anti-HBV treatments [27-33]. It has been shown that AEs of CHB are accompanied by increased T cell responses to hepatitis B core and e antigens (HBcAg & HBeAg) [34]. There is also evidence that HBcAg and/or HBeAg may represent important "targets" for immune-mediated viral clearance [3-7, 35, 36]. Moreover, naturally arising forkhead transcription factor Foxp3 (forkhead box p3)-expressing $CD4^+CD25^+$ regulatory T (T_{reg}) cells are thought to have important role in the control of infectious diseases [37] including viral infections such as viral hepatitis B [38-40] and C [41] as well as dengue fever [42], while their role in modulating spontaneous AEs of CHB and in influencing the outcome of anti-HBV treatments remains unexplored.

To address this issue, we employed the SYFPEITHI scoring system [43] to predict HLA class II-restricted epitope peptides on HBcAg overlapping with HBeAg for the construction of peptide-HLA class II tetramers. These tetramers were used to measure HBcAg-specific T_{reg} cell-frequencies ($T_{reg}f$) in patients with spontaneous AEs and during anti-HBV treatments.

Materials and Methods

Study subjects

Since August 2002 when it was included in the health insurance coverage in Taiwan, a nationwide therapeutic trial of anti-HBV treatments for CHB patients was initiated. This trial included: regimen I, treatment with 18-month oral 3TC (100 mg per day, GlaxoSmithKline) for CHB patients with AEs; regimen II, the same but 6-month for HBeAg positive patients; and regimen III, 12-month long-acting IFN-α [PEGASYS® (Roche) i.e., peginterferon α-2a, 180μg subcutaneous injection once a week] for HBeAg positive and anti-HBe positive patients respectively with elevation of serum alanine aminotransferase (ALT) level greater than two times upper limit of normal (ULN = 40 U/L) with liver histology showing positive staining of nuclear and/or cytoplasmic HBcAg. For 3TC-resistant patients, oral ADV (10 mg per day, GlaxoSmithKline) add-on and/or replacement of 3TC were administrated for 24 months (regimen IV). All these regimens were recommended by the Bureau of National Health Insurance of Taiwan.

A total of 188 CHB patients who fulfilled the criteria for the treatment visited Chi Mei Medical Center (Tainan, Taiwan) were enrolled for the therapeutic trial. All were perinatally-acquired chronic HBV infection as they had a clear history of familial HBV infection and were seropositive for HBsAg, HBeAg and HBV-DNA for at least 6 months. Cases seropositive for hepatitis C virus, hepatitis D virus and human immunodeficiency virus (HIV) were excluded in this study. Among them, 40 cases (Supplementary Table; HBV 1-36 & HBV 45-48) were recruited for further immunological analysis. Besides, eight 3TC naive CHB patients (Supplementary Table; HBV 37-44) receiving oral ETV therapy (Bristol-Myers Squibb, 0.5 mg per day for 18 months) were enrolled for the study purpose. During this period, another 14 perinatally-acquired CHB patients with HLA-A2 who developed spontaneous AEs without treatment were included as controls. Informed consent was obtained from each of all study subjects and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and approved by the Ethical Committee of the Institute.

AE or acute flare of CHB was defined as an abrupt elevation of serum ALT level greater than 5 × ULN, i.e., ALT >200 U/L; and without superinfection with any other hepatotropic viruses [26, 34]. Serum HBV-DNA was quantified by HBV RealQuant™ PCR assay kit (General Biologicals Corporation, Hsin-

Tzu, Taiwan). The detection limit of HBV-DNA by this assay was 3000 copies/mL (=600 IU/mL) of genomic HBV-DNA. HBV genotyping was carried out by line probe assay using commercially available kit (INNO-LiPA HBV Genotyping, Innogenetics N.V., Belgium) according to the manufacturer's instructions. HLA typing on peripheral blood mononuclear cells (PBMCs) was performed using standard serological techniques (Terasaki HLA Tissue Typing Trays, One Lambda Inc., Canoga Park, CA). DRB1*0101 (DR1) and DRB1*0401 (DR4) typings were conducted with DNA typing method (Micro SSP™, HLA class II DNA Typing Trays DRB1*01 and DRB1*04, One Lambda). Some patients with HLA class II haplotypes other than DR1 and DR4 were included as mutual controls to ensure the specificity of the peptide-HLA class II tetramer staining assay. Because only HLA class II tetramers of DRB1*0101 and DRB1*0401 molecules were currently commercially available, we focused on patients with these two HLA-DR alleles.

In this trial, SR to the treatment was defined as normalization of serum ALT level, loss of serum HBV-DNA by HBV RealQuant™ PCR assay, and seroconversion of HBeAg to its antibody (anti-HBe) sustained for 12 months after the end of treatment. A transient normalization of serum ALT level (biochemical response) or loss of HBeAg and HBV-DNA (virological response) followed by a relapse either after the end of treatment or during therapy was defined as partial response (PR). Nonresponse (NR) was defined as the lack of either a biochemical or a virological response to the therapy [44].

Preparation of HLA-A2-HBcAg 18-27 tetrameric complexes

HLA-A2-restricted epitope peptide HBcAg 18-27 FLPSDFFPSV, an immunodominant cytotoxic T lymphocyte (CTL) epitope on HBcAg, is currently the most widely used epitope for assaying CTL activities in HBV patients [28, 31, 33, 45, 46]. We used HBcAg 18-27-HLA-A2 tetrameric complexes in this study to monitor CTL activities for the correlation with T_{reg} *f* in HLA-A2 patients. The tetrameric complexes were prepared as described previously.

Utilization of the SYFPEITHI scoring system to predict epitope peptides on HBcAg for the construction of peptide-HLA class II tetramers

Epitopes on HBcAg restricted by HLA class II antigens, DRB1*0101 and DRB1*0401 molecules have not been identified in humans, yet. To ensure the success of tetramer synthesis with 15-mer peptides on HBcAg overlapping with HBeAg, the SYFPEITHI scoring system [43] was employed to predict possible epitope peptides that can bind to DRB1*0101 and DRB1*0401 molecules (Table 1 and Supplementary Fig 1). The 17 peptides with 15-mer amino acid residues used in this study were arbitrarily designed, of which, P2 (HBcAg 16-30)

Table 1. SYFPEITHI scores of 15-mer HBcAg and HBsAg peptides on DRB1*0101 and DRB1*0401 molecules.

PP No.	A.A. residues	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	SYFPEITHI SCORE DRB1*0101	SYFPEITHI SCORE DRB1*0401
1	HBcAg 1-15	M	D	I	D	P	Y	K	E	F	G	A	T	V	E	L	1	1
2	HBcAg 16-30[a]	L	S	F	L	P	S	D	F	F	P	S	V	R	D	L	16	20
3	HBcAg 31-45	L	D	T	A	S	A	L	Y	R	E	A	L	E	S	P	16	18
4	HBcAg 46-60	E	H	C	S	P	H	H	T	A	L	R	Q	A	I	L	8	6
5	HBcAg 61-75	C	W	G	E	L	M	T	L	A	T	W	V	G	V	N	18	6
6	HBcAg 76-90	L	E	O	P	A	S	R	D	L	V	V	S	Y	V	N	8	1
7	HBcAg 91-105	T	N	M	G	L	K	F	R	Q	L	L	W	F	H	I	2	12
8	HBcAg 106-120	S	C	L	T	F	G	R	E	T	V	I	E	Y	L	V	7	1
9	HBcAg 121-135	S	F	G	V	W	I	R	T	P	P	A	Y	R	P	P	17	3
10	HBcAg 131-145	A	Y	R	P	P	N	A	P	I	L	S	T	L	P	E	0	12
11	HBcAg 136-150	N	A	P	I	L	S	T	L	P	E	T	T	V	V	R	14	14
12	HBcAg 151-165	R	R	G	R	S	P	R	R	R	T	P	S	P	R	R	9	7
13	HBcAg 129-143[b]	P	P	A	Y	R	P	P	N	A	P	I	L	S	T	L	28	16
14	HBcAg 130-144	P	A	Y	R	P	P	N	A	P	I	L	S	T	L	P	14	6
15	HBcAg 166-183	R	R	S	Q	S	P	R	R	R	R	S	Q	S	R	E	0	7
16	HBsAg 85-100[b]	L	T	T	V	P	A	A	P	P	A	S	T	N	R	Q	26	0
17	HBsAg124-138[a]	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	Q	2	26

a. Peptides P2 and P17 were used for the construction of DRB1*0401 tetramers, and b, Peptides P13 and P16, for DRB1*0101 tetramers.

PP No, peptide number; A. A. amino acid. .

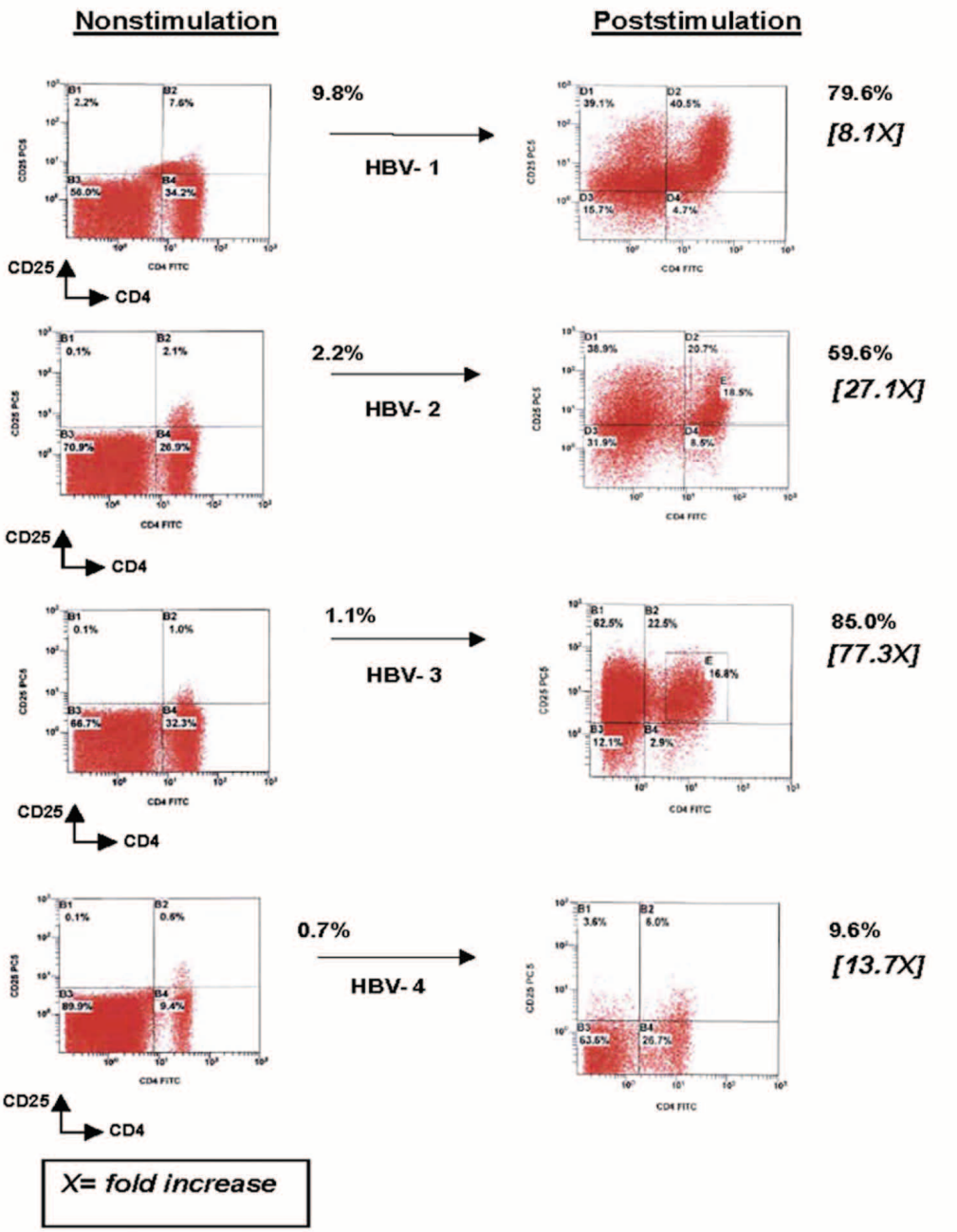

Figure 1. Vigorous *in vitro* expansion of Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells responding to IL-2 and viral peptides. The results of four experiments that reproducibly demonstrated vigorous expansion of $CD4^+CD25^+$ T_{reg} cells stimulated *in vitro* with IL-2 and viral peptides. Preferential increase in $CD25^+$ T cells was noted after short-term culture.

and P13 (HBcAg 129-143) had the highest SYFPEITHI scores 20 and 28 on DRB1*0101 and DRB1*0401 molecules respectively. They were used for the construction of each of HLA class II tetramers and were purchased from Beckman Coulter Inc. (San Diego, CA, USA; Kit Lot No.C505190 and C510066, respectively). Tetramers of 15-mer peptides derived from HBsAg, P16 (HBsAg 85-100) and P17 (HBsAg 124-138) were used as assay controls for P13 and P2 tetramers respectively.

In vitro short-term culture to enhance peptide-HLA class II tetramer staining efficiency

Emerging evidence indicates that $CD4^+CD25^+$ T_{reg} cells may expand *in vitro* by stimulation with foreign antigen plus interleukin-2 (IL-2) [47-49]. T cell activation state and temperature may affect the

efficiency of tetramer staining [50-52]. Because low percentages of T_{reg} cells in PBMCs and very small number of liver-infiltrating T lymphocytes (LITs) can be isolated from liver biopsy specimens, a strategy of *in vitro* short-term culture was used to solve this problem as described before (Supplementary Fig 2) [33]. LITs were collected as described previously [53]. Generally, this method gives rise to at least 5- to 15-fold or more increase in total numbers of T_{reg} cells compared to that obtained directly from PBMCs or LITs *ex vivo* [33, 54].

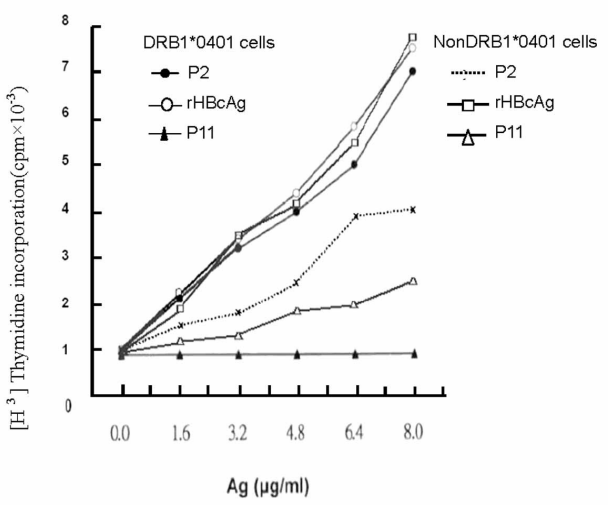

Figure 2. HLA class II-restricted epitope peptides predicted by the SYFPEITHI scoring system correlate with proliferation assays. In terms of [H³] thymidine incorporation, a significant proliferation of affinity-purified $CD4^+CD25^+$ T_{reg} cells from the DRB1*0401 patient responding to both peptide P2 (Table 1) and rHBcAg, but undetectable proliferation to peptide P11 (Fig 1) with an SYFPEITHI score "0" on DRB1*0401 molecule.

Proliferative assays of PBMCs with and without depletion of $CD4^+CD25^+$ T_{reg} cells

$CD4^+CD25^+$ T_{reg} cells were depleted from PBMCs of seven CHB patients without anti-HBV treatment by affinity-purified method using Dynal® $CD4^+CD25^+$ T_{reg} kit (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's instructions. Also, another eight patients without depletion of T_{reg} cells were examined as controls. In 96-well flat-bottomed microculture trays (Nunc, Roskilde, Denmark), T_{reg} cells depleted and undepleted PBMCs were plated in triplicate at a density of 1×10^5 cells per well in complete medium (RPMI 1640 medium supplemented with 5% heat-inactivated bovine serum albumin, 25 mM Hepes, 2 mM L-glutamine, 1% sodium pyruvate, 0.05 mM 2-mercaptoethanol, and antibiotics; all from Gibco Laboratories, Grand Island, NY) coculturing with 1 μg/mL recombinant HBcAg (rHBcAg) and then subjected to a 7-day proliferative assay as described previously [34]. Blocking assays using mouse anti-human program death receptor 1 (PD1) (hybridoma clone 192106; R & D Systems Inc., Minneapolis, MN, USA), mouse anti-human cytotoxic T lymphocyte associated antigen-4 (CTLA-4) or CD152 (hybridoma clone BN13; Immunotech Co, Marseille, France) and mouse anti-human transforming growth factor-β1 (TGF-β1; hybridoma clone 9016; R & D Systems Inc., Minneapolis, MN, USA) were performed in the eight subjects without depletion of T_{reg} cells by coculturing PBMCs with each monoclonal antibody at a concentration of 10 ng/mL in complete medium with addition of 1 μg/mL rHBcAg.

Flow cytometry analysis

For flow cytometry analysis, cells were processed on a Beckman Coulter EPICS Altra Hypersort System (Beckman Coulter Inc., CA) and analyzed by EXPO2 software (Beckman Coulter). Cell surface markers included staining with antibodies anti-PD1, anti-CD152, anti-CD4, anti-CD8, and anti-CD25 (Immunotech Co, Marseille, France). Intracellular staining (ICS) including cytokines was conducted using mouse anti-human Foxp3 (Biolegend, San Diego, CA), anti-human IL-2 (Immunotech Co, Marseille, France), anti-human IL-4 (Immunotech Co, Marseille, France), and anti-human IL-10 (Serotec, Oxford, UK). ICS for TGF-β1 was conducted by an indirect immunocytochemical method using mouse anti-human TGF-β1 monoclonal antibody (R&D System Inc., Minneapolis, MN, USA) as the first antibody and goat anti-mouse IgG as the second antibody (Chemicon International, Temecula, CA).

Statistical analysis

Statistical analysis was carried out with the SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney U test or Wilcoxon signed rank test was used to compare two unpaired or paired nonparametric data respectively. For two groups of independent categorical data, chi-square test was used for the comparison. Difference with a P value less than 0.05 was considered statistically significant.

Results

Vigorous in vitro expansion of Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells responding to HBcAg peptides in combination with IL-2

$CD4^+CD25^+$ T_{reg} cells from PBMCs of CHB

patients express Foxp3 (Supplementary Fig 3). They expand vigorously *in vitro* stimulated by HBcAg and/or its peptides plus IL-2 (Fig 1). In *ex vivo* condition without stimulation, less than 10% of $CD4^+$ cells from PBMCs of all four CHB patients expressing CD25 molecule, comparable with the report in normal naive mice that CD25 molecule is expressed by 5-10% of $CD4^+$ T cells and 5% of $CD4^+CD8^+$ mature thymocytes [55]. After short-term *in vitro* culturing with HBcAg peptides plus IL-2, vigorous expansion of $CD4^+CD25^+$ T_{reg} cells was consistently detected. Moreover, a significant proportion of these T_{reg} cells express IL-2 (Supplementary Fig 4a), which is considered essential for the survival of mature Foxp3 T_{reg} cells and its signal is critical for the maintenance of T_{reg} cells in the periphery [47-49]. IL-2 secreted by T_{reg} cells themselves may further stimulate cell proliferation via an autocrine manner.

HLA class II-restricted epitope peptides predicted by the SYFPEITHI scoring system correlate with proliferation assays

A significant proliferation of $CD4^+CD25^+$ T_{reg} cells from one DRB1*0401 patient responded to both peptide P2 (Table 1) and rHBcAg in terms of

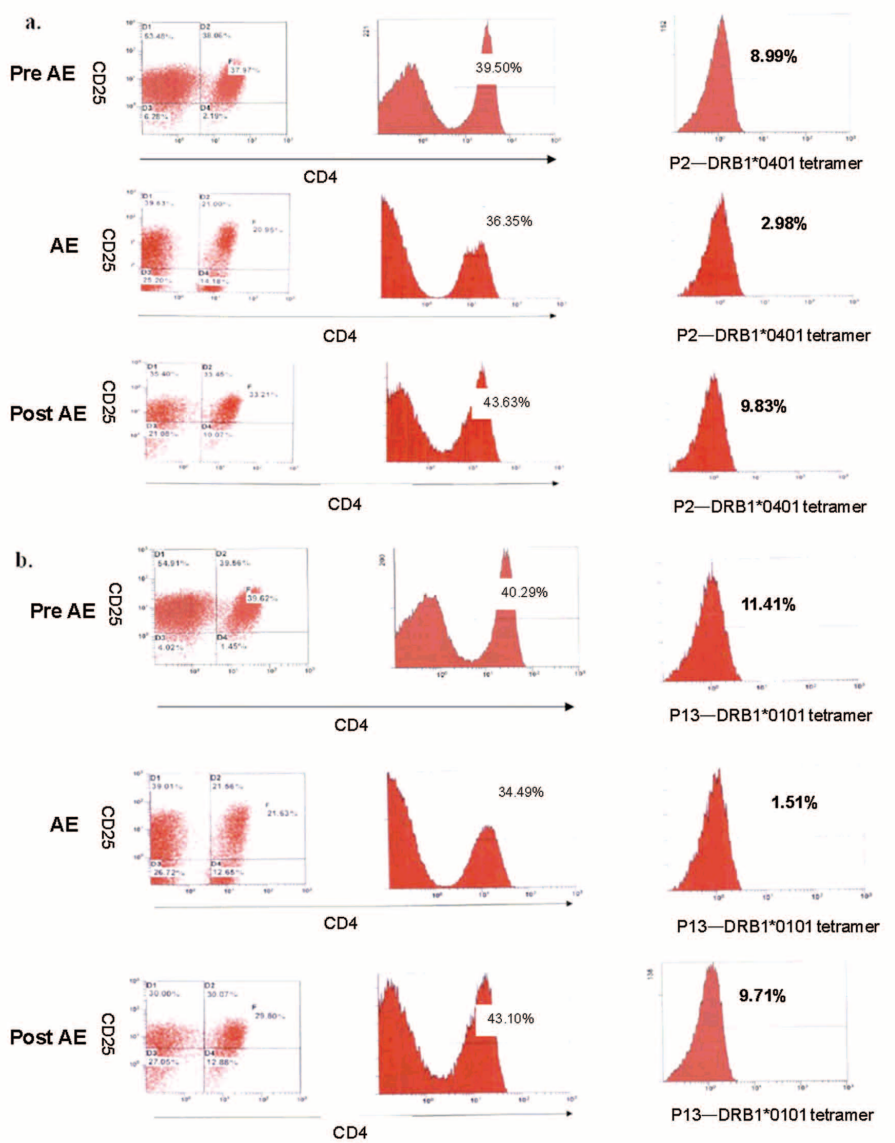

Figure 3. HBcAg-specific T_{reg} *f* declines during spontaneous AEs. Using P2-DRB1*0401 and P13-DRB1*0101 tetramers for assaying HBcAg-specific T_{reg} *f*, CHB patients with DRB1*0401 (3a) and DRB1*0101 (3b) respectively who developed spontaneous AEs without treatment. P2-specific T_{reg} *f*: 8.99% (pre-AE phase)→2.98% (Peak AE phase)→9.83% (Post-AE phase). P13-specific T_{reg} *f*: 11.41% (pre-AE phase)→1.51% (peak AE phase)→9.71% (Post-AE phase).

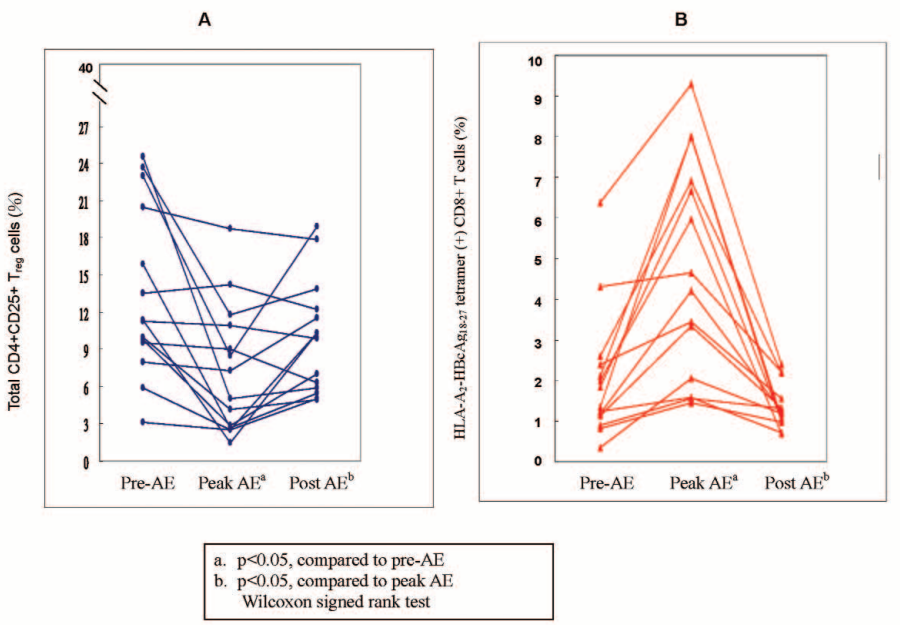

Figure 4. HBcAg-specific T_{reg} *f* declines during spontaneous AE accompanied by increased HBcAg-specific CTL frequencies (Tc*f*). The evolution of total percentages of $CD4^+CD25^+$ T_{reg} cells (A, total T_{reg} *f*) and HBcAg-specific Tc*f* in terms of HLA-A2-HBcAg 18-27 tetramer staining $CD8^+$ T cells (B) in PBMCs of 14 CHB patients with HLA-A2 during spontaneous AEs. Total T_{reg} *f*: total percentages of $CD4^+CD25^+$ T_{reg} cells in PBMCs after short-term *in vitro* culture stimulated with rHBcAg plus IL-2 for one week (Fig 2). Tc*f*: percentages of HLA-A2-HBcAg 18-27 tetramer staining $CD8^+$ T cells in PBMCs *ex vivo* without stimulation (Fig 6).

[H^3] thymidine incorporation was detected (Fig 2), but low or nearly undetectable proliferation to peptide P11 (HBcAg11-25 with an SYFPEITHI score "0" on DRB1*0401 molecule (Supplementary Fig 1). These data were compatible with a higher SYFPEITHI score "20" on DRB1*0401 molecule for the P2 peptide, which was used for the construction of peptide-DRB1*0401 tetramer. Likewise, similar results were obtained from DRB1*0101 patients responding to peptide P13 (Table 1) which could be fit for the construction of peptide-DRB1*0101 tetramer. Both of which were specific for DRB1*0401 and DRB1*0101 cells, respectively (Supplementary Fig 5).

HBcAg-specific T_{reg} f declines during spontaneous AEs accompanied by increased HBcAg-specific CTL frequencies

Using P2-DRB1*0401 and P13-DRB1*0101 tetramers for assaying HBcAg-specific T_{reg} *f*, CHB patients with DRB1*0401 (Fig 3a) and DRB1*0101 (Fig 3b) respectively who developed spontaneous AEs without treatment revealed that P2- and P13-specific T_{reg} *f* tended to fall from pre-AE phase to peak AE phase associated with a decrease of total percentages of $CD4^+CD25^+$ T_{reg} cells (total T_{reg} *f*). HBcAg-specific T_{reg} *f* subsequently tended to rise in post AE phase. Reversely, HBcAg 18-27-HLA-A2 tetramer staining $CD8^+$ CTL frequencies (Tc*f*) increased from pre-AE phase to peak AE phase and in turn decreased in post AE phase in CHB patients

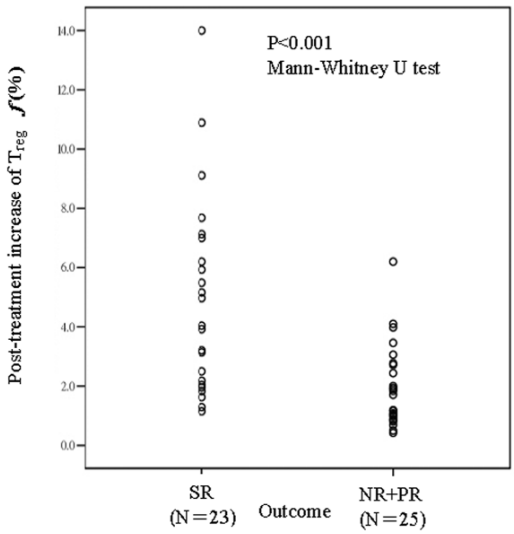

Figure 5. More post-treatment increase of T_{reg} *f* correlates with a higher sustained remission rate to anti-HBV treatments. Dot plot shows T_{reg} *f* of 48 patients receiving anti-HBV therapy including 23 cases with sustained remission (SR) and 25 cases with partial response (PR) plus non-response (NR) (Supplementary Table). More post-treatment increase of T_{reg} *f* correlated with a higher SR rate to the therapy (P<0.001).

with HLA-A2 (Supplementary Fig 6). As a whole, the evolutional changes of $T_{reg}f$ (Fig 4a) and Tc*f* (Fig 4b) of 14 CHB patients with HLA-A2 who developed spontaneous AEs without treatment, revealed that total $T_{reg}f$ declined to the lowest level accompanied by increase of HBcAg-specific Tc*f* to the highest level at peak AE phase ($P<0.05$, Wilcoxon signed rank test).

More post-treatment increase in HBcAg-specific $T_{reg}f$ correlates with a higher sustained remission rate to anti-HBV treatments

Figure 5 shows the changes $T_{reg}f$ of the 48 CHB patients who received anti-HBV treatments (Clinical features, treatment outcomes and each datum are listed in supplementary Table). Overall, more post-treatment increase in HBcAg-specific and/or total $T_{reg}f$ correlates with a higher SR rate to the therapy (SR vs. NR+PR, $P<0.001$, Mann-Whitney U test). The total $T_{reg}f$ of patients with SR to IFN-α injection therapy decreased initially and in turn increased after the end of treatment (data not shown). Figure 6 demonstrates the increase of P2-DRB1*0401 tetramer staining $CD4^+CD25^+$ T_{reg} cells in two DRB1*0401 patients with SR to anti-HBV therapy: (a) 3TC with ADV add-on therapy and (b) ETV therapy respectively.

Depletion of $CD4^+CD25^+$ T_{reg} cells from PBMCs enhances proliferation to rHBcAg

$CD4^+CD25^+$ T_{reg} cells from PBMCs and liver-infiltrating lymphocytes of CHB patients also express CD152, PD1 and TGF-β1 (Supplementary Fig 7, 8, 9 & 4b, respectively). They exhibit

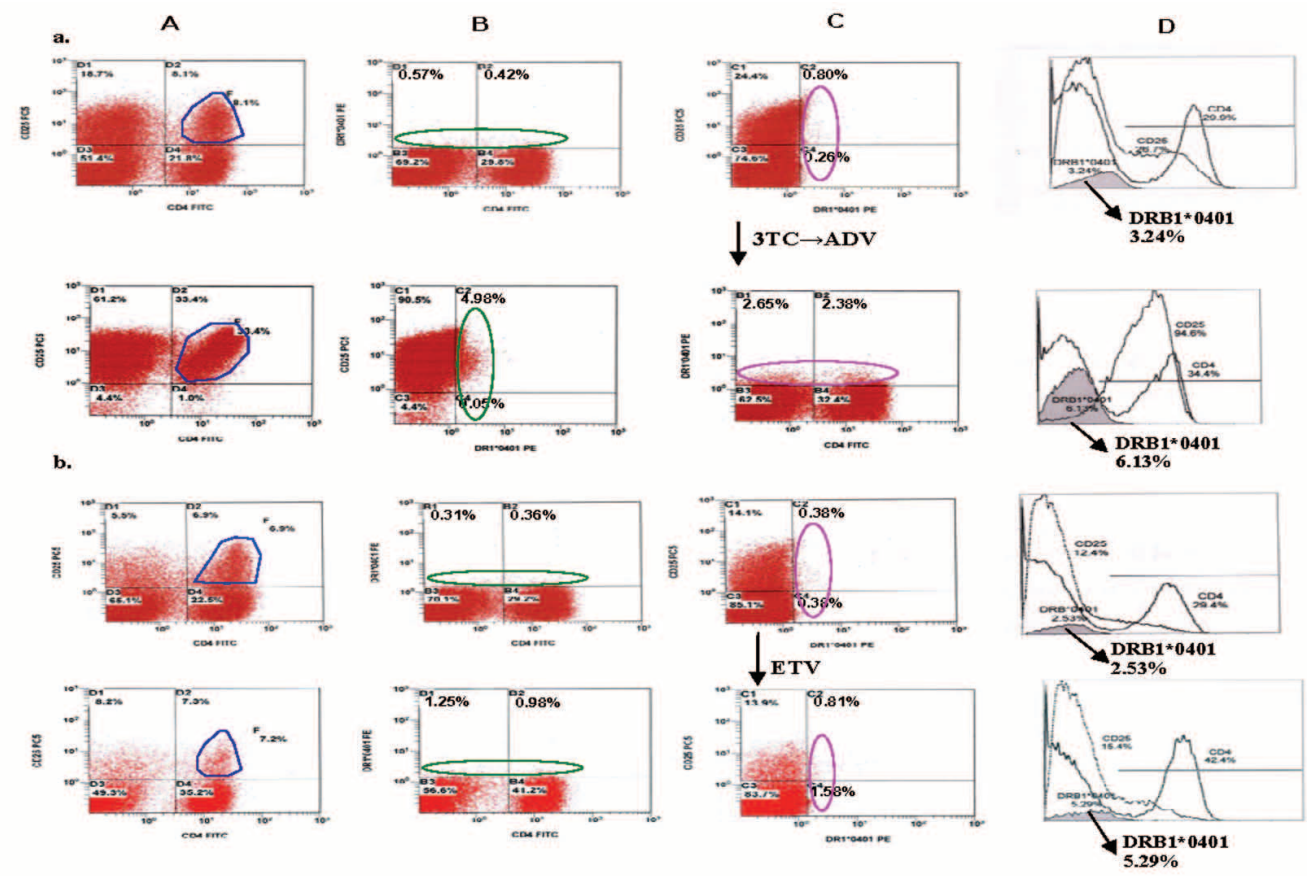

Figure 6. Post-treatment increase of T_{reg} *f* in sustained remission cases. Flow cytometry analysis on PBMCs *ex vivo* without in vitro stimulation demonstrated post-treatment increase of T_{reg} *f* in two DRB1*0401 patients with SR to anti-HBV therapy: (a) 3TC with ADV add-on therapy and (b) ETV therapy, respectively. Panels A, B & C are dot plots of the flow cytometry analysis; panel D shows the overlay plots.

suppression on PBMCs proliferation to rHBcAg. Depletion of $CD4^+CD25^+$ T_{reg} cells from PBMCs significantly enhanced proliferation to rHBcAg in terms of increased stimulation indexes (Fig 7a vs. 7b, P<0.001, Mann-Whitney U test). This enhancement could be reproduced by the addition of antibodies including anti-CD152 (Fig 7c), anti-PD1 (Fig 7d) and anti-TGF-β1 (Fig 7e) to the cultures respectively, but was abolished by IL-4, IL-10 and TGF-β1 (Supplementary Fig 10). One possible mechanism for the enhanced rHBcAg-stimulated proliferation by anti-CD152 might be via the blocking of CTLA-4 engagement leading to the inhibition of TGF-β1 secretion which is thought to be a mediator of $D4^+CD25^+$ T_{reg} cells for suppressor effectors' function [56]. It is reported that *in vivo* blockade of the inhibitory molecule PD-1 can restore the function of exhausted $CD8^+$ T cells during chronic viral infection, specifically in HIV patients [57]. The mechanism of our *in vitro* experiments showing the enhancement of PBMCs proliferation to rHBcAg by PD-1 blockade requires further investigation.

Discussion

T_{reg} cells are a heterogeneous group of naturally arising and/or peripherally induced antigen-specific $CD4^+$ or $CD8^+$ T cells that secrete immunoregulatory cytokines with the ability to inhibit the proliferation of other cells. By the conventional definition, T_{reg} cells are anergic to foreign antigen stimulation, non-proliferating nature, low IL-2 secretion and requiring cell-cell contact for the function of suppressor effectors [58-62].

In current study, we focused on HBcAg-specific $CD4^+CD25^+$ T_{reg} cells. Our results were obviously contradictory to these features, showing that HBcAg-specific T_{reg} cells from CHB patients expanded vigorously in response to antigen stimulation and were proliferating to viral peptides plus IL-2. They also express IL-2. The reason might be attributed to the fact that all the study subjects were perinatally-acquired CHB patients. Their $D4^+CD25^+$ T_{reg} cells had been long-term priming with HBV antigens including HBcAg and its epitope peptides presented by HLA molecules on virus-infected hepatocytes or other antigen presenting cells since perinatal period. Hence those T_{reg} cells those were capable of expansion and proliferation in response to the stimulation with HBcAg and its peptides plus IL-2 is not totally unexpected.

Our results are compatible with reports that MHC class II tetramers may identify peptide-specific human $CD4^+$ T cells proliferating to viral antigens [63, 64], and with that T_{reg} cell population can be induced and expanded by foreign antigen [47, 48]. The SYFPEITHI scoring system was successfully employed in this study to predict epitope peptides on HBcAg overlapping with HBeAg restricted by DRB1*0401 and DRB1*0101 molecules. Without treatment, HBcAg-specific T_{reg} *f* declined during spontaneous AEs accompanied by increased HBcAg-specific Tc*f*. In contrast, more post-treatment increase of T_{reg} *f* correlated with a higher SR rate to the therapy in CH-B patients receiving anti-HBV treatments. The former could be theoretically reconciled with the suppressor effectors' function of T_{reg} cells.

Decline of HBcAg-specific T_{reg} *f* may relieve HBcAg/HBeAg-specific CTLs from suppression by T_{reg} cells and elicit an overwhelming CTL-mediated hepatocytolysis manifested clinically by a rapid increase of serum ALT levels (usually more than 5- to 10- fold ULN), i.e., an AE episode which may lead to

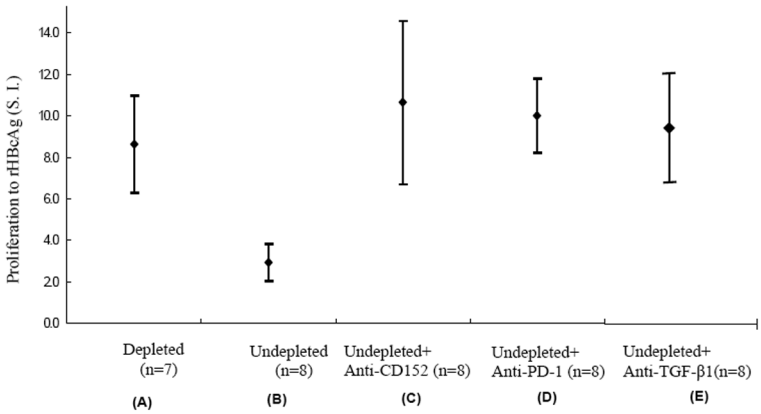

Figure 7. $CD4^+CD25^+$ T_{reg} cell depletion enhances PBMCs Proliferation to rHBcAg. PBMCs of 7 CHB patients with depletion of $CD4^+CD25^+$ T_{reg} cells (A), and of 8 patients without depletion (B) were assayed for proliferation to rHBcAg. A significant enhancement of proliferation was noted with depletion $CD4^+CD25^+$ T_{reg} cells from PBMCs (A vs. B, P<0.001). This enhancement could be reproduced by addition of anti-CD152 (C), anti-PD1 (D) and anti-TGF-β1 (E) antibodies. rHBcAg: recombinant HBcAg, SI: stimulation index.

necrosis of HBV-infected hepatocytes resulting in the clearance of HBV-DNA and loss of viral antigens. Spontaneous AEs may evolve to spontaneous HBeAg seroconversion with hepatitis remission [1, 4-7, 26, 65]. The logic for the latter might be interpreted as that long-term suppression of viral replication with nucleoside analogues may downregulate antigen presentation on hepatocyte HLA molecules because of the decrease in serum HBV-DNA levels and in synthesis of HBV antigens [5, 7, 35]. This mimicked the scenario of the late phase of spontaneous AEs with HBeAg seroconversion to anti-HBe and ALT normalization along with increase of T_{reg} *f*. Higher post-treatment increase in T_{reg} *f* suggests more complete inhibition of HBV replication by anti-HBV treatments, specifically by nucleoside analogues, as evidenced by the fact that serum ALT levels of most patients on continuing anti-HBV treatments are kept normal unless the appearance of resistant mutant viruses [21, 25].

Women who are chronic carriers of HBV often infect infants in the perinatal or postnatal periods, whereas intrauterine infection is much less common [66, 67]. Neonates born to HBV carrier mothers may be immunologically tolerant to viral proteins to which they were exposed *in utero* [66, 67]. The vast majority of untreated infants born to HBeAg-positive mothers become infected, and more than 90% of them become chronic HBV carriers, the so-called "perinatally-acquired" chronic HBV infection [1, 7, 66-68]. Those carriers are characterized by active viral replication, low or normal serum ALT levels, positive serum HBeAg with high HBV-DNA levels while necrosis of hepatocytes is rarely found on liver biopsy specimens. This patient group is referred to as on the immune tolerance phase which can last for long and variable periods until adulthood or older age [1, 3, 5, 7, 68].

It is shown in an HBeAg transgenic mice model that a function of HBeAg, cross reactive with HBcAg, may be to induce immunologic tolerance *in utero* [69]. The coexistence of tolerance to HBc/HBe T-cell determinants and anti-HBc production *in vivo* is comparable to the immunologic status of neonates born to carrier mothers [72]. It is conceivable that HBeAg cross-reactive with HBcAg may be viewed as a self-antigen recognized by HBcAg-specific T_{reg} cells maintaining perinatally-acquired CHB patients in the immune tolerance status despite of high serum HBV-DNA levels and seropositive for HBeAg [1-7, 65-68]. Decline of HBcAg-specific T_{reg} *f* results in spontaneous AEs.

In summary, our results indicate that HBcAg-specific T_{reg} cells can expand in response to stimulation with HBcAg and its peptides plus IL-2. They exhibit suppressor activity and play a crucial role in modulating spontaneous AEs and in successful anti-HBV treatments. Collectively, these data could be integrated together as a model in patients with AEs who received anti-HBV treatments illustrated in Figure 8. It will be of clinical interest to manipulate T_{reg} cells during anti-HBV treatments for "perinatally-acquired" chronic HBV infection [70, 71].

Acknowledgments

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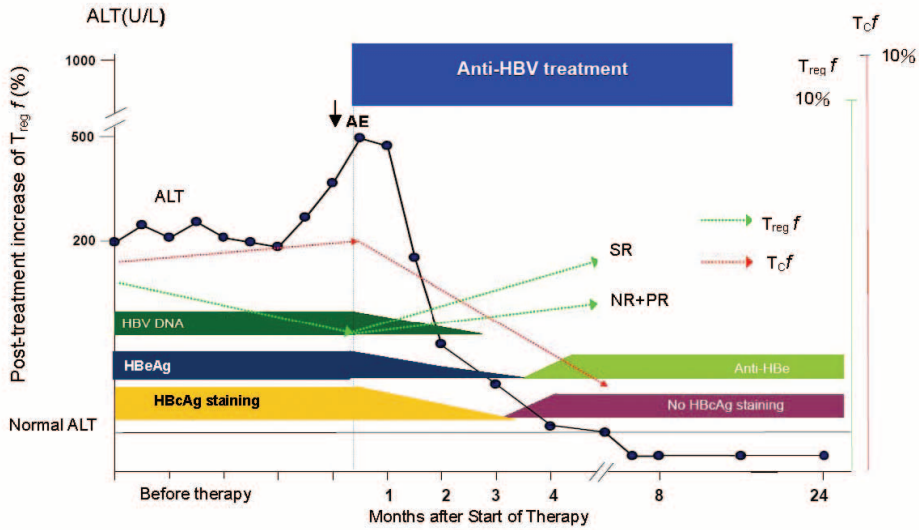

Figure 8. A model for evolutional changes of T_{reg} *f* and Tc*f* in CHB patients with AEs receiving anti-HBV treatments. Evolutional changes of T_{reg} *f* and Tc*f* detected on PBMCs in CHB patients with AEs receiving anti-HBV treatments in relation to serum levels of HBV DNA, ALT, HBeAg and anti-HBe as well as hepatocyte HBcAg expression. T_{reg} *f*: Total percentages of $CD4^+CD25^+$ T_{reg} cells detected on PBMCs *ex vivo* without short-term in vitro culture or stimulation. Tc*f*: Percentages of HLA-A2-HBcAg 18-27 tetramer staining $CD8^+$ T cells in CHB patients with HLA-A2 on PBMCs *ex vivo* without short-term *in vitro* culture or stimulation.

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Supplementary Table 1. Characteristics of CHB patients with anti-HBV treatments.

Patient	Genotype	Age	Gender	DR typing	HLA-A2	ALT (U/L)		HBeAg		Loss of HBV-DNA‡		$T_{reg}f$(%) #		Tcf(%)		Treatment §	Outcome□
						Pre-Tx	End	Pre-Tx	End			Pre-T	End	Pre-T	End		
HBV-1	B	26	M	DRB1*0401	HLA-A2	367	22	+	-		Yes	1.40	2.55			3TC	SR
HBV-2	B	58	M			1399	23	+	-		Yes	0.27	2.31			3TC	SR
HBV-3	B	25	F		HLA-A2	423	129	+	+	No		1.12	1.54	5.60	1.77	3TC	NR
HBV-4	B	38	M			477	54	+	+	No		1.33	2.02			3TC	NR
HBV-5	B	31	M	DRB1*0101	HLA-A2	367	20	+	-		Yes	2.12	9.80	7.55	1.12	3TC	SR
HBV-6	B	18	M			480	28	+	-		Yes	0.99	7.19			3TC	SR
HBV-7	B	38	M			2087	765	+	+	No		1.27	3.20			3TC	NR
HBV-8	B	32	M			419	18	+	-		Yes	1.33	3.51			3TC	SR
HBV-9	B	23	M			397	60	+	+	No		4.43	5.59			3TC	PR
HBV-10	B	25	F	DRB1*0401	HLA-A2	350	37	+	-		Yes+	2.50	7.99	3.77	1.23	3TC	SR
HBV-11	B	26	M			857	35	+	-		Yes	1.11	4.25			3TC	SR
HBV-12	B	24	M			1057	21	+	+	No		7.14	10.2			3TC	PR
HBV-13	B	31	M			214	62	+	+	No		3.90	5.74			3TC	NR
HBV-14	B	42	M	DRB1*0401	HLA-A2	348	482	+	+	No		4.45	7.91	1.99	1.11	3TC	NR
HBV-15	B	22	M			326	50	+	+	No		8.10	9.15			3TC	NR
HBV-16	B	37	F			290	31	+	+	No		5.54	6.41			3TC	PR
HBV-17	B	36	M			624	20	+	-		Yes	1.49	6.65			3TC	SR
HBV-18	B	26	M			580	31	+	+	No		2.59	4.50			3TC	PR
HBV-19	B+C	39	M		HLA-A2	1461	130	+	+	No		1.51	2.59	2.33	0.15	3TC	NR
HBV-20	B+C	42	M			614	24	+	+	No		2.27	6.25			3TC	PR
HBV-21	B+C	24	M			2289	24	+	-		Yes	0.88	4.80			3TC	SR
HBV-22	B+C	22	F	DRB1*0401		165	105	+	+	No		1.77	2.75			3TC	NR
HBV-23	C	45	M			334	64	+	+	No		5.51	6.70			3TC	NR
HBV-24	C	38	M		HLA-A2	1598	79	+	-	No	+	7.73	10.5	6.05	4.97	3TC	PR
HBV-25	C	35	F			1769	47	+	+	No		4.49	4.99			3TC	NR
HBV-26	C	33	F			527	631	+	+	No		5.55	7.99			3TC	NR
HBV-27	C	28	M			561	10	+	-		Yes	2.71	6.75			3TC	SR
HBV-28	C	31	M			336	21	+	-		Yes	2.11	13.00			3TC	SR
HBV-29	C	54	M	DRB1*0401	HLA-A2	2638	40	+	-		Yes	1.00	10.11	3.08	1.00	3TC	SR
HBV-30	C	32	M			366	148	+	-	No		4.59	7.31			3TC→ADV	NR
HBV-31	C	15	M			587	22	+	-		Yes	4.05	9.02			3TC→ADV	SR
HBV-32	C	22	M	DRB1*0401		391	23	+	-		Yes	3.24	6.13			3TC→ADV	SR
HBV-33	C	27	M			312	38	+	+	No		2.55	3.42			3TC→ADV	PR
HBV-34	B	40	M			2589	116	+	+	No		3.11	4.82			3TC+ADV	PR
HBV-35	C	62	F			2775	25	+	-		Yes	3.37	9.30			3TC+ADV	SR

Supplementary Table 1. Characteristics of CHB patients with anti-HBV treatments.

Patient	Genotype	Age	Gender	DR typing	HLA-A2	ALT (U/L)		HBeAg		Loss of HBV-DNA‡		$T_{reg}f$(%) #		Tcf(%) *		Treatment §	Outcome□
HBV-36	B	45	M	DRB1*0101	HLA-A2	571	15	+	-		Yes	2.73	9.86	5.23	1.02	3TC→ADV	SR
HBV-37	B	42	M			1147	24	+	-		Yes	0.79	2.42			ETV	SR
HBV-38	B	36	M			1762	31	+	-		Yes	0.9	14.9			ETV	SR
HBV-39	B+C	22	M	DRB1*0401		1465	28	+	-		Yes	2.53	5.29			ETV	SR
HBV-40	C	38	M			306	35	+	+	No		5.2	9.3			ETV	PR
HBV-41	C	25	M	DRB1*0101	HLA-A2	293	26	+	-		Yes	0.19	2.02	3.42	0.31	ETV	SR
HBV-42	B	44	F	DRB1*0401	HLA-A2	269	55	+	+	No		3.3	9.5	4.11	3.90	ETV	PR
HBV-43	C	55	M			428	40	+	+	No		3.1	5.1			ETV	PR
HBV-44	C	29	M			524	33	-	+		Yes	2.11	4.07			ETV	SR
HBV-45	B	31	M	DRB1*0401		114	62	+	+	No		4.22	5.03			IFN-α	NR
HBV-46	B	61	M			131	117	+	-	No	+	3.55	4.56			IFN-α	PR
HBV-47	C	42	M			121	26	+	-		Yes	1.12	4.33			IFN-α	SR
HBV-48	C	46	F	DRB1*0101	HLA-A2	184	28	-	+		Yes	1.49	3.99	7.11	2.03	IFN-α	SR

‡ By HBV RealQuant™ PCR assay at the end of treatment.

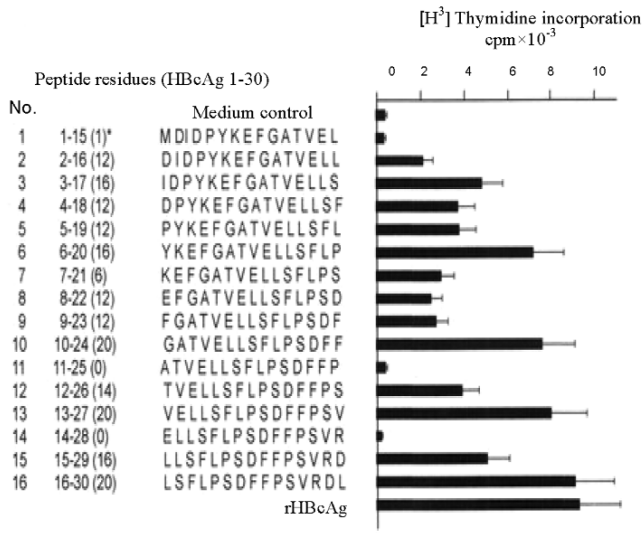
Total percentages of $CD4^+CD25^+$ Treg cells detected on PBMCs *ex vivo* from patients negative for DRB1*0101 or DRB1*0401; and percentages of P2-DRB1*0401- or P13-DRB1*0101-tetramer staining (+) $CD4^+CD25^+$ Treg cells for DRB1*0401 and DRB1*0101 cases respectively.

*Percentages of HLA-A2-HBcAg 19-27 tetramer staining $CD8^+$ T cells on PBMCs *ex vivo* without stimulation.

§ 3TC: lamivudine; ADV: adefovir dipivoxil; ETV: entecavir; IFN-α: interferon-α; 3TC→ADV: treatment shifted from 3TC to ADV

□SR: sustained remission; NR: non-response; PR: partial response

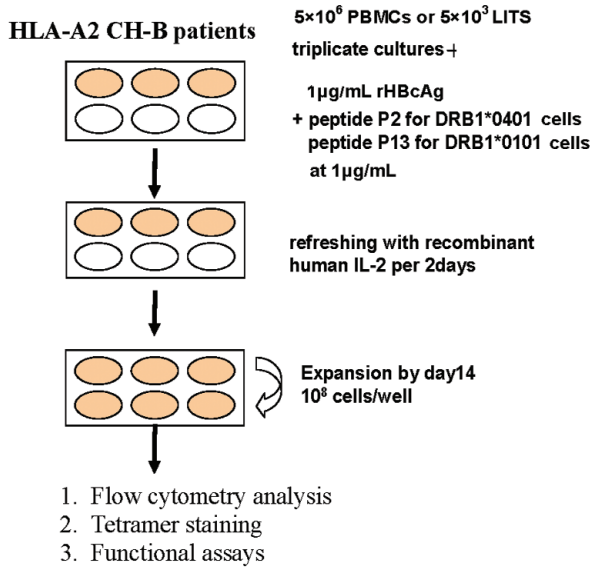
Pre-Tx: pre-treatment; End: at the end of treatment



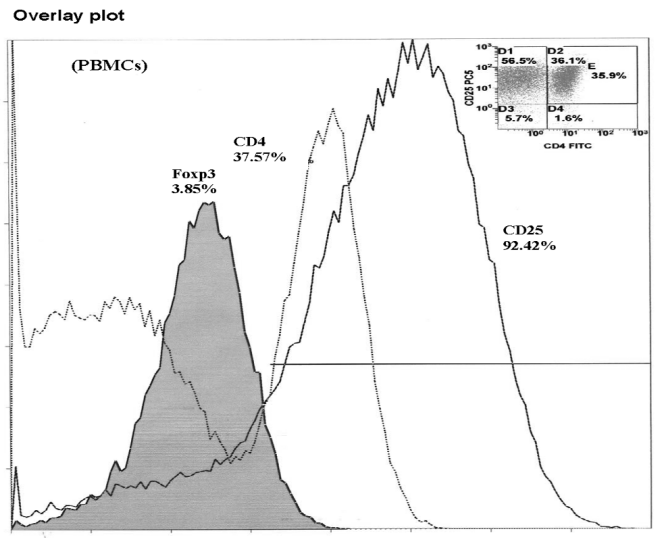
***SYFPEITHI score on DRB1*0401molecule**

The prediction is based on published motifs (pool sequencing, natural ligands) and takes into consideration the amino acids in the anchor and auxiliary anchor positions, as well as other frequent amino acids. The score is calculated according to the following rules: The amino acids of a certain peptide are given a specific value depending on whether they are anchor, auxiliary anchor or preferred residue. Ideal anchors will be given 10 points, unusual anchors 6-8 points, auxiliary anchors 4-6 points and preferred residues 1-4 points. Amino acids that are regarded as having a negative effect on the binding ability are given values between -1 and -3.[42]

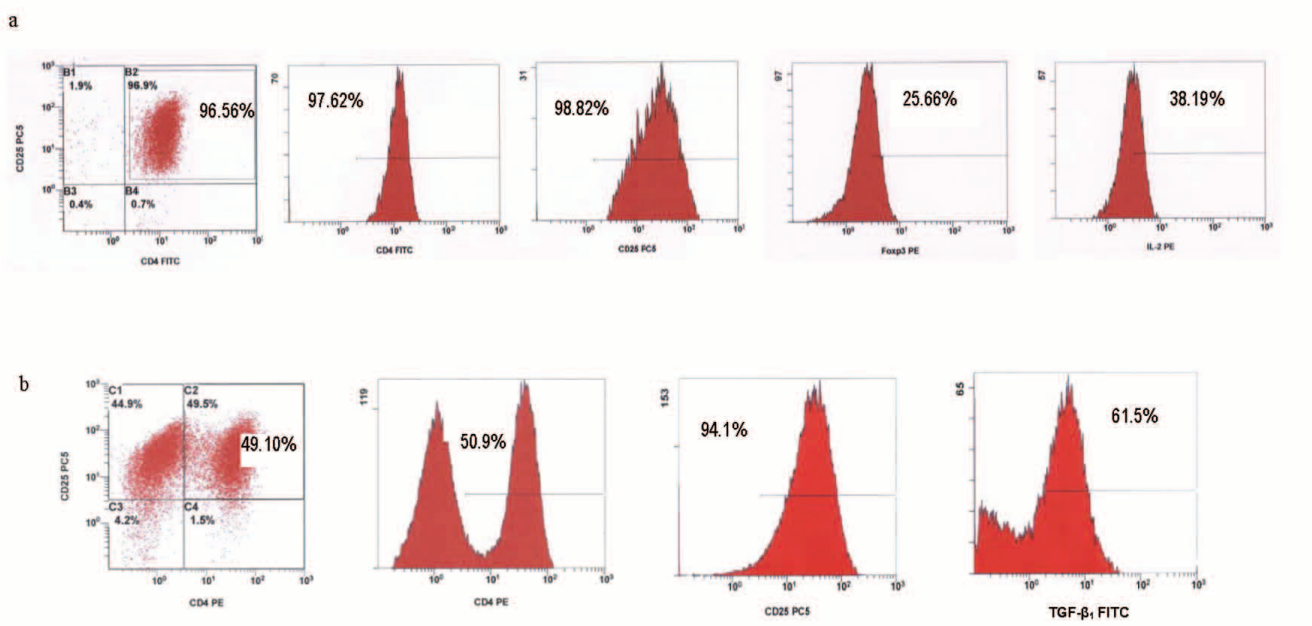
Supplementary Figure 1. SYFPEITHI scores of overlapping 15-mer amino acid peptides at HBcAg 1-16 on DRB1*0401 molecule. Each score was obtained by input the 15-mer peptide sequence into the SYFPEITHI score website (free access via: http://www.syfpeithi.de). These 16 peptides with continuous amino acids (aa) sequences, each of which has one aa overlapping. Peptide No.16, HBcAg 15-30 is equal to the peptide P2 shown in Table 2 with an SYFPEITHI score "20" on DRB1*0401 molecule. It can induce proliferation of DRB1*0401 cells in terms of $[H^3]$ thymidine incorporation (shown in Fig 2), while peptide No.11 with an SYFPEITHI score "0" cannot.



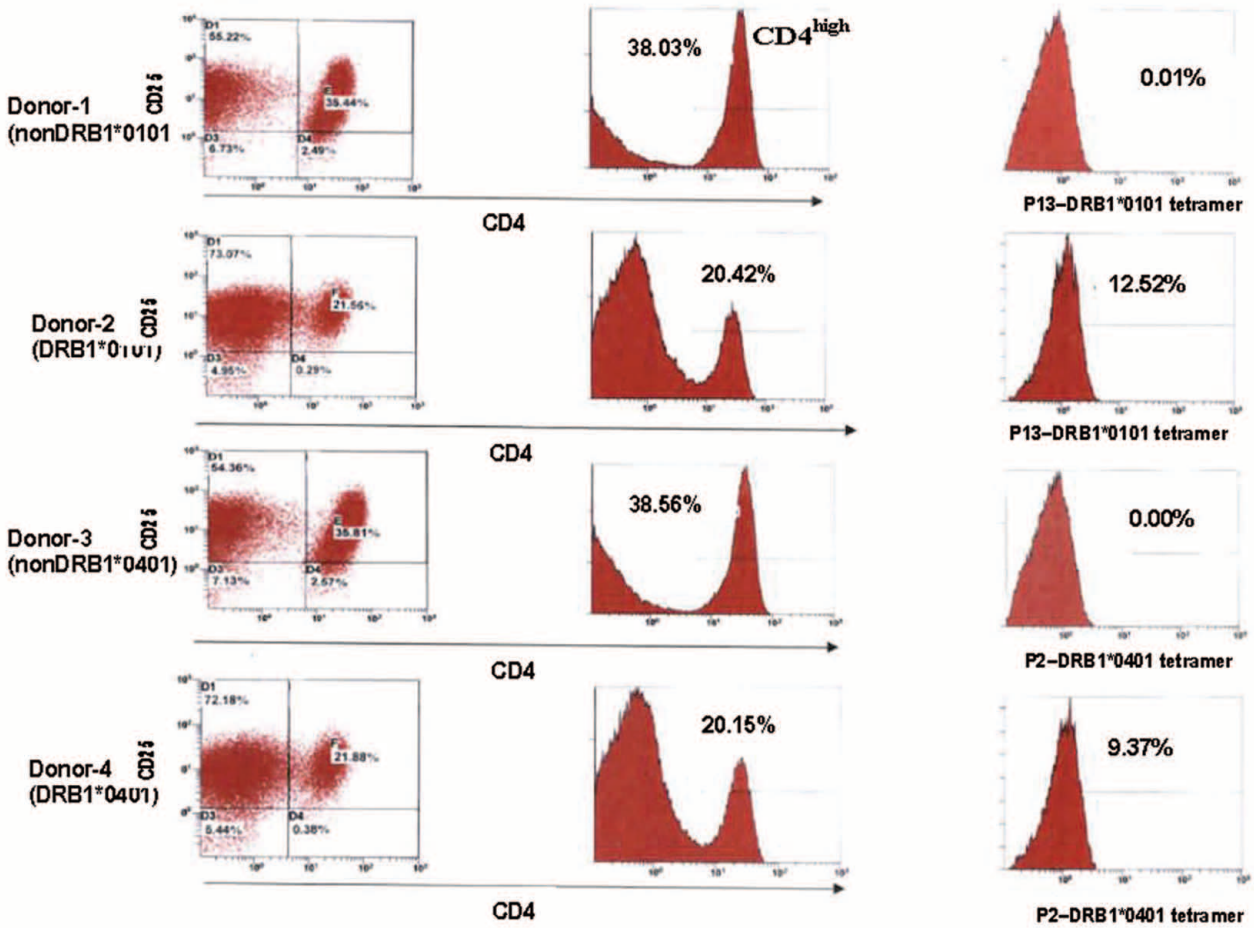
Supplementary Figure 2. The strategy for in vitro short-term culture to activate and expand HBcAg-specific $CD4^+CD25^+$ T_{reg} cells. $CD4^+CD25^+$ T_{reg} cells from peripheral blood mononuclear cells (PBMCs) or liver-infiltrating T lymphocytes (LITs) of CHB patients with HLA-A2 were expanded by stimulation with peptide P2 (for DRB1*0401 cells) or P13 (for DRB1*0101 cells) plus IL-2 and recombinant HBcAg (rHBcAg).



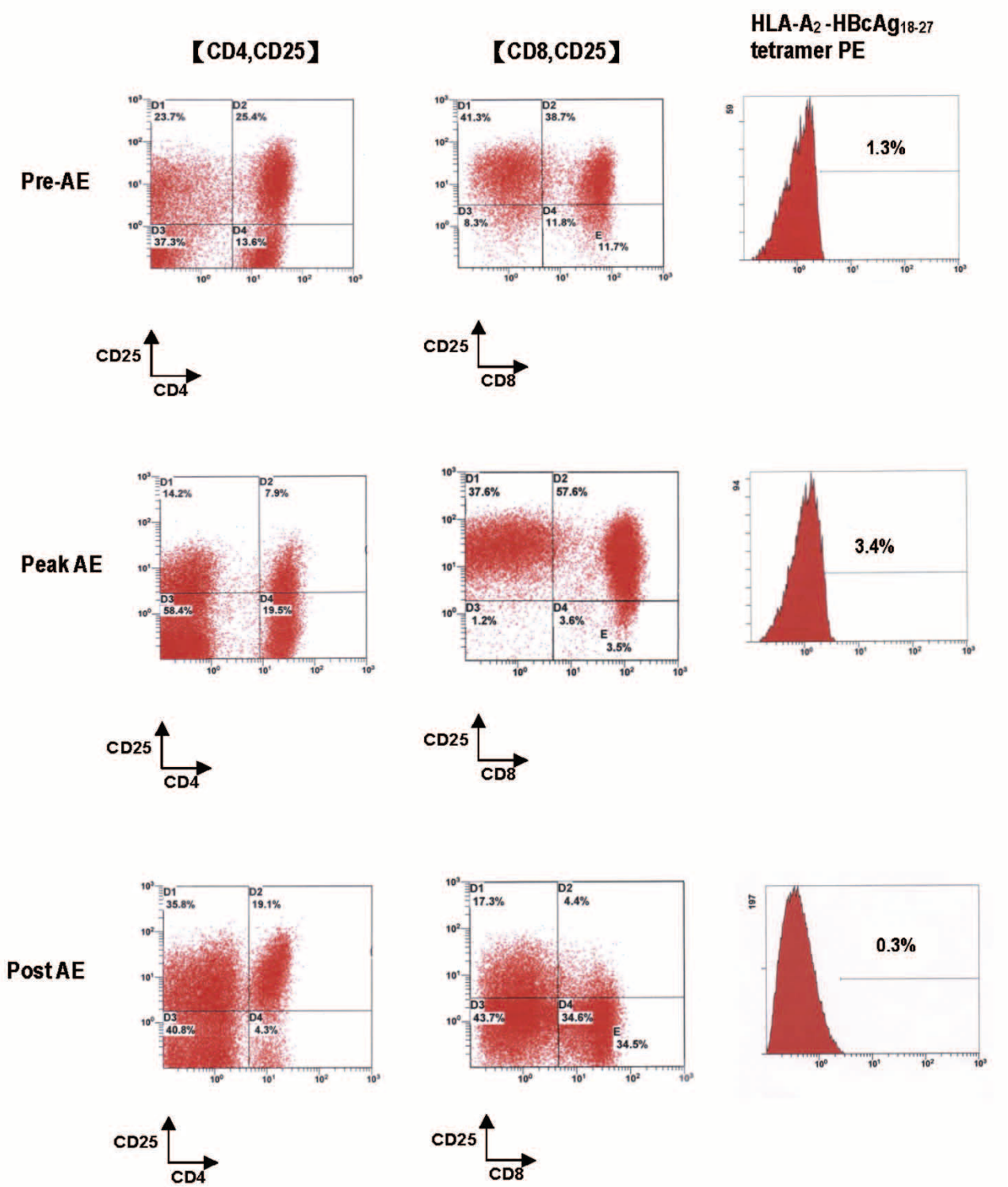
Supplementary Figure 3. $CD4^+CD25^+$ T_{reg} cells from PBMCs of CHB patients express Foxp3. Overlay plot of $CD4^+CD25^+$ T_{reg} cells expanded from PBMCs (by short-term *in vitro* culture) of one CHB patient showed 3.85% of them were positive for Foxp3 staining.



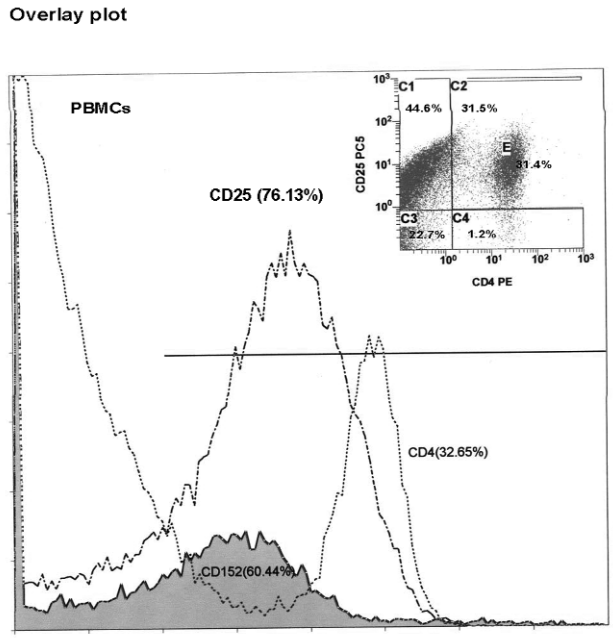
Supplementary Figure 4. $CD4^+CD25^+$ T_{reg} cells from PBMCs of CHB patients express (a) IL-2, and (b) TGF-β1. A significant proportion (up to 38.15%) of $CD4^+CD25^+$ T_{reg} cells sorted from PBMCs after short-term *in vitro* culture of one CHB patient (shown in Figure 1, experiment 3) express IL-2 (a), and TGF-β1 (b, up to 61.5%).



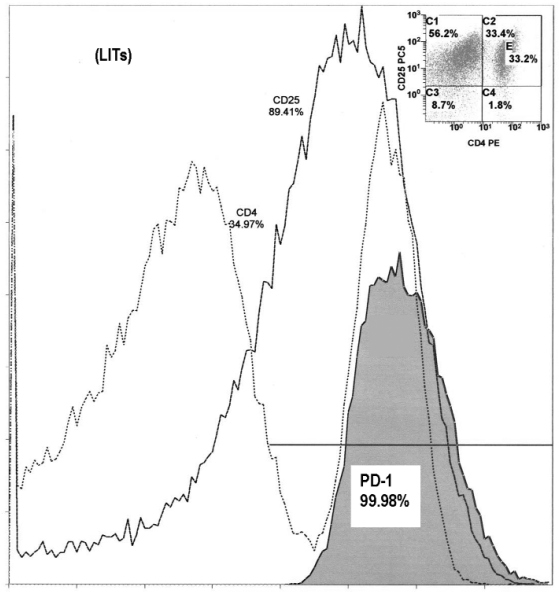
Supplementary Figure 5. Control assays for P13- and P2-specific tetramers. As assay controls, flow cytometry analysis of P13- or P2-specific tetramer staining $T_{reg}f$ on PBMCs after short-term *in vitro* culture from CHB patients negative for DRB1*0101 (donor 1, panel 1) and negative for DRB1*0401 (donor 3, panel 3) reveals a staining percentage 0.01% and 0.00% of total $CD4^+CD25^+$ T_{reg} cells respectively, ascertaining that the tetramer staining is specific for DRB1*0101 and DRB1*0401 T_{reg} cells, respectively. Likewise only background staining can be detected in cells from these same cases stained with P16-DRB1*0101 and P17-DRB1*0401 tetramers respectively (data not shown), suggesting the tetramer staining is specific to HBcAg.



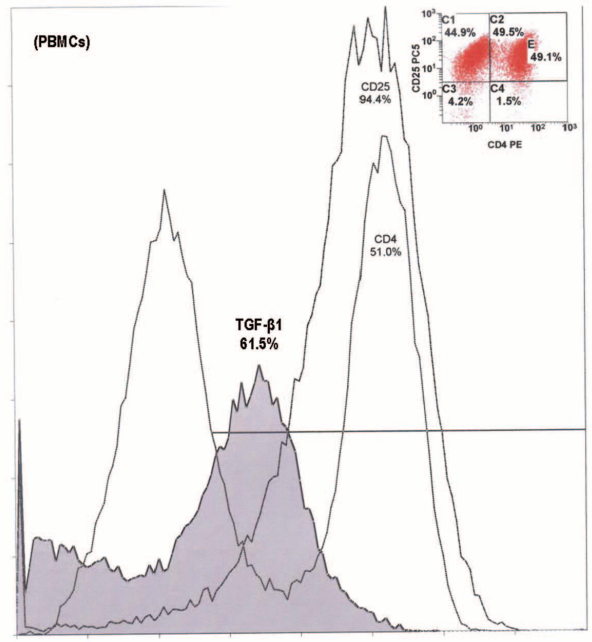
Supplementary Figure 6. Total $CD4^+CD25^+$ T_{reg} cells (total T_{reg} *f*) decreased during spontaneous AEs accompanied by increased HBcAg-specific CTL frequencies. A representative case with HLA-A2 receiving serial follow-up study showed a decrease of total percentages of $CD4^+CD25^+$ T_{reg} cells, from pre-AE phase to peak AE phase accompanied by an increase of HBcAg 18-27 tetramer-staining $CD8^+$ CTL frequencies (Tc*f*).



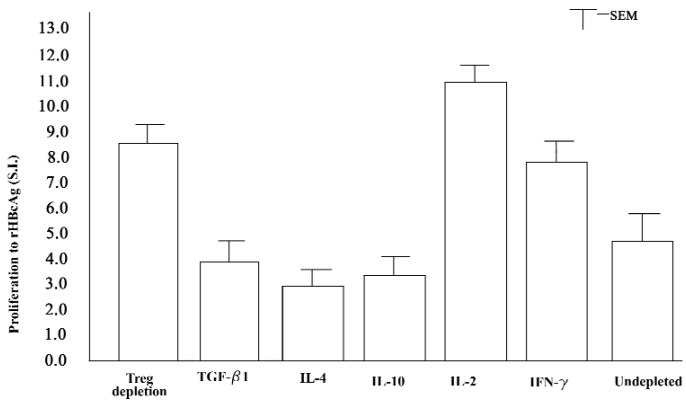
Supplementary Figure 7. Flow cytometry analysis of CD152 staining on PBMCs. Overlay plot of CD152 staining on $CD4^+CD25^+$ T_{reg} cells from PBMCs after *in vitro* short-term culture of one patient with AE without treatment revealed 60.44% of them were positive for CD152.



Supplementary Figure 8. Flow cytometry analysis of PD1 staining on $CD4^+CD25^+$ T_{reg} cells expanded from LITs. Overlay plot of PD1 staining on $CD4^+CD25^+$ T_{reg} cells from LITs expanded by short-term *in vitro* culture of one CHB patient before IFN-α injection therapy showed that nearly total (99.98%) of activated LITs express PD1 antigen.



Supplementary Figure 9. Flow cytometry analysis of intracellular TGF-β1 staining on $CD4^+CD25^+$ T_{reg} cells expanded from PBMCs. Overlay plot of TGF-β1 staining on $CD4^+CD25^+$ T_{reg} cells from PBMCs expanded by short-term *in vitro* culture of one CHB patient with AE before ETV therapy showed that 61.5% of the activated PBMCs express TGF-β1.



Supplementary Figure 10. Effects of cytokines IL-2, IL-4, IL-10, IFN-γ, and TGF-β1 on $CD4^+CD25^+$ T_{reg} cell depleted PBMCs in response to rHBcAg. In the proliferative assays of PBMCs with depletion of $CD4^+CD25^+$ T_{reg} cells in response to rHBcAg (1 μg/mL) with the addition of recombinant human IL-2 (10 ng/mL, R&D Systems, Inc., Minneapolis, MN), IL-4 (10 pg/mL, PeproTech EC Ltd., London, UK), IL-10 (10 pg/mL, R&D Systems), IFN-γ (10 pg/mL, R&D Systems), and TGF-β1 (10 pg/mL, R&D Systems). The results showed that the enhancement of PBMCs proliferation to rHBcAg by the depletion of $CD4^+CD25^+$ T_{reg} cells was abolished by IL-4, IL-10, and TGF-β1, but further stimulated by IL-2 and IFN-γ.

References

1. Chen DS. From hepatitis to hepatoma: lessons from type B viral hepatitis. *Science* 1993; **262**: 369-70.
2. Purcell RH. Hepatitis viruses: changing patterns of human disease. *Proc Natl Acad Sci U S A* 1994; **91**: 2401-6.
3. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev* 2005; **5**: 215-29.
4. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Ann Rev Immunol* 1995; **13**: 29-60.
5. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-45.
6. Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-39.
7. Ganem D, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-29.
8. Greenberg HB, Pollard RB, Lutwick LI, Gregory PB, Robinson WS, Merigan TC. Effect of human leukocyte interferon on hepatitis B virus infection in patients with chronic active hepatitis. *N Engl J Med* 1976; **295**: 517-22.
9. Desmyter J, De Groote J, Desmet VJ, Billiau A, Ray MB, Bradburne AF, *et al.* Administration of human fibroblast interferon in chronic hepatitis-B infection. *Lancet* 1976; **2**: 645-7.
10. Sullivan SD, Veenstra DL, Chen PJ, Chang TT, Chuang WL, Tsai C, *et al.* Cost-effectiveness of peginterferon alpha-2a compared to lamivudine treatment in patients with hepatitis B e antigen positive chronic hepatitis B in Taiwan. *J Gastroenterol Hepatol* 2007; **22**: 1494-9.
11. Hoofnagle JH, di Bisceglie AM. The treatment of chronic viral hepatitis. *N Engl J Med* 1997; **336**: 347-56.
12. Peters MG, Hann Hw H, Martin P, Heathcote EJ, Buggisch P, Rubin R, *et al.* Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology* 2004; **126**: 91-101.
13. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521-31.
14. Chien RN, Liaw YF, Chen TC, Yeh CT, Sheen IS. Efficacy of thymosin alpha1 in patients with chronic hepatitis B: a randomized, controlled trial. *Hepatology* 1998; **27**: 1383-7.
15. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, *et al.* Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* 2005; **365**: 123-9.
16. Lai CL, Leung N, Teo EK, Tong M, Wong F, Hann HW, *et al.* A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. *Gastroenterology* 2005; **129**: 528-36.
17. Liaw YF. Hepatitis flares and hepatitis B e antigen seroconversion: implication in anti-hepatitis B virus therapy. *J Gastroenterol Hepatol* 2003; **18**: 246-52.
18. Chien RN, Lin CH, Liaw YF. The effect of lamivudine therapy in hepatic decompensation during acute exacerbation of chronic hepatitis B. *J Hepatol* 2003; **38**: 322-7.
19. Yousefi-Mashhour M, Mansour-Ghanaei F, Foroutan H, Ghofrani H, Purrasuli Z, Joukar F. Efficacy of lamivudine therapy on decompensated liver cirrhosis due to chronic hepatitis B. *Hep Mon* 2007; **7**: 77-81.
20. Zoulim F. Entecavir: a new treatment option for chronic hepatitis B. *J Clin Virol* 2006; **36**: 8-12.
21. Hollinger FB, Lau DT. Hepatitis B: the pathway to recovery through treatment. *Gastroenterol Clin North Am* 2006; **35**: 895-931.
22. Jarvis B, Faulds D. Lamivudine. A review of its therapeutic potential in chronic hepatitis B. *Drugs* 1999; **58**: 101-41.
23. Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; **47**: 451-60.
24. Yokosuka O, Omata M, Imazeki F, Ito Y, Okuda K. Hepatitis B virus RNA transcripts and DNA in chronic liver disease. *N Engl J Med* 1986; **315**: 1187-92.
25. Hussain M, Lok AS. Mutations in the hepatitis B virus polymerase gene associated with antiviral treatment for hepatitis B. *J Viral Hepat* 1999; **6**: 183-94.
26. Perrillo RP. Acute flares in chronic hepatitis B: the natural and unnatural history of an immunologically mediated liver disease. *Gastroenterology* 2001; **120**: 1009-22.
27. Marinos G, Naoumov NV, Williams R. Impact of complete inhibition of viral replication on the cellular immune response in chronic hepatitis B virus infection. *Hepatology* 1996; **24**: 991-5.
28. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, *et al.* Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* 1998; **102**: 968-75.
29. Hultgren C, Weiland O, Milich DR, Sallberg M. Cell-mediated immune responses and loss of hepatitis B e-antigen (HBeAg) during successful lamivudine and famciclovir combination therapy for chronic replicating hepatitis B virus infection. *Clin Infect Dis* 1999; **29**: 1575-7.
30. Liaw YF, Tsai SL, Chien RN, Yeh CT, Chu CM. Prednisolone priming enhances Th1 response and efficacy of subsequent lamivudine therapy in patients with chronic hepatitis B. *Hepatology* 2000; **32**: 604-9.
31. Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, *et al.* Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. *Hepatology* 2001; **33**: 963-71.
32. Tsai SL, Sheen IS, Chien RN, Chu CM, Huang HC, Chuang YL, *et al.* Activation of Th1 immunity is a common immune mechanism for the successful treatment of hepatitis B and C: tetramer assay and therapeutic implications. *J Biomed Sci* 2003; **10**: 120-35.
33. Lin CL, Tsai SL, Lee TH, Chien RN, Liao SK, Liaw YF. High frequency of functional anti-YMDD and -mutant cytotoxic T lymphocytes after in vitro expansion correlates with successful response to lamivudine therapy for chronic hepatitis B. *Gut* 2005; **54**: 152-61.
34. Tsai SL, Chen PJ, Lai MY, Yang PM, Sung JL, Huang JH, *et al.* Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. *J Clin Invest* 1992; **89**: 87-96.
35. Chu CM, Liaw YF. Intrahepatic distribution of hepatitis B surface and core antigens in chronic hepatitis B virus infection. Hepatocyte with cytoplasmic/membranous hepatitis B core antigen as a possible target for immune hepatocytolysis. *Gastroenterology* 1987; **92**: 220-5.
36. Tsai SL, Chen MH, Yeh CT, Chu CM, Lin AN, Chiou FH, *et al.* Purification and characterization of a naturally processed hepatitis B virus peptide recognized by CD8+ cytotoxic T lymphocytes. *J Clin Invest* 1996; **97**: 577-84.
37. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005; **6**: 353-60.
38. Stoop JN, van der Molen RG, Baan CC, van der Laan LJ, Kuipers EJ, Kusters JG, *et al.* Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 2005; **41**: 771-8.

39. Franzese O, Kennedy PT, Gehring AJ, Gotto J, Williams R, Maini MK, *et al.* Modulation of the CD8+-T-cell response by CD4+CD25+ regulatory T cells in patients with hepatitis B virus infection. *J Virol* 2005; **79**: 3322-8.
40. Xu D, Fu J, Jin L, Zhang H, Zhou C, Zou Z, *et al.* Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. *J Immunol* 2006; **177**: 739-47.
41. Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, *et al.* An immunomodulatory role for CD4 (+) CD25 (+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-71.
42. Luhn K, Simmons CP, Moran E, Dung NT, Chau TN, Quyen NT, *et al.* Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection. *J Exp Med* 2007; **204**: 979-85.
43. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 1999; **50**: 213-9.
44. Chien RN, Yeh CT, Tsai SL, Chu CM, Liaw YF. Determinants for sustained HBeAg response to lamivudine therapy. *Hepatology* 2003; **38**: 1267-73.
45. Maini MK, Boni C, Ogg GS, King AS, Reignat S, Lee CK, *et al.* Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. *Gastroenterology* 1999; **117**: 1386-96.
46. Maini MK, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, *et al.* The role of virus-specific CD8 (+) cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* 2000; **191**: 1269-80.
47. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 2005; **6**: 1219-27.
48. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 2005; **6**: 1142-51.
49. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 2005; **6**: 1152-9.
50. Reichstetter S, Ettinger RA, Liu AW, Gebe JA, Nepom GT, Kwok WW. Distinct T cell interactions with HLA class II tetramers characterize a spectrum of TCR affinities in the human antigen-specific T cell response. *J Immunol* 2000; **165**: 6994-8.
51. Cameron TO, Cochran JR, Yassine-Diab B, Sekaly RP, Stern LJ. Cutting edge: detection of antigen-specific CD4+ T cells by HLA-DR1 oligomers is dependent on the T cell activation state. *J Immunol* 2001; **166**: 741-5.
52. Nolte-'t Hoen EN, Amoroso MG, Veenstra J, Grosfeld-Stulemeyer MC, van Eden W, Broeren CP, *et al.* Effector and regulatory T cells derived from the same T cell clone differ in MHC class II-peptide multimer binding. *Eur J Immunol* 2004; **34**: 3359-69.
53. Liaw YF, Lee CS, Tsai SL, Liaw BW, Chen TC, Sheen IS, *et al.* T-cell--mediated autologous hepatocytotoxicity in patients with chronic hepatitis C virus infection. *Hepatology* 1995; **22**: 1368-73.
54. Tsai SL, Lee TH, Chien RN, Liao SK, Lin CL, Kuo GC, *et al.* A method to increase tetramer staining efficiency of CD8+ T cells with MHC-peptide complexes: therapeutic applications in monitoring cytotoxic T lymphocyte activity during hepatitis B and C treatment. *J Immunol Methods* 2004; **285**: 71-87.
55. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**: 345-52.
56. Chen W, Jin W, Wahl SM. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4(+) T cells. *J Exp Med* 1998; **188**: 1849-57.
57. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, *et al.* PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**: 350-4.
58. Read S, Powrie F. CD4 (+) regulatory T cells. *Curr Opin Immunol* 2001; **13**: 644-9.
59. von Herrath MG, Harrison LC. Antigen-induced regulatory T cells in autoimmunity. *Nat Rev* 2003; **3**: 223-32.
60. Nagler-Anderson C, Bhan AK, Podolsky DK, Terhorst C. Control freaks: immune regulatory cells. *Nat Immunol* 2004; **5**: 119-22.
61. Kronenberg M, Rudensky A. Regulation of immunity by self-reactive T cells. *Nature* 2005; **435**: 598-604.
62. Fathman CG, Lineberry NB. Molecular mechanisms of CD4+ T-cell anergy. *Nat Rev* 2007; **7**: 599-609.
63. Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4 (+) T cells proliferating in response to influenza A antigen. *J Clin Invest* 1999; **104**: R63-7.
64. Novak EJ, Masewicz SA, Liu AW, Lernmark A, Kwok WW, Nepom GT. Activated human epitope-specific T cells identified by class II tetramers reside within a CD4high, proliferating subset. *Int Immunol* 2001; **13**: 799-806.
65. Hoofnagle JH, Shafritz DA, Popper H. Chronic type B hepatitis and the "healthy" HBsAg carrier state. *Hepatology* 1987; **7**: 758-63.
66. Beasley RP, Hwang LY. Postnatal infectivity of hepatitis B surface antigen-carrier mothers. *J Infect Dis* 1983; **147**: 185-90.
67. Beasley RP, Trepo C, Stevens CE, Szmuness W. The e antigen and vertical transmission of hepatitis B surface antigen. *Am J Epidemiol* 1977; **105**: 94-8.
68. Lok AS. Chronic hepatitis B. *N Engl J Med* 2002; **346**: 1682-3.
69. Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci U S A* 1990; **87**: 599-603.
70. Bluestone JA. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev* 2005; **5**: 343-9.
71. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev* 2007; **7**: 585-98.