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HBcAg-specific CD4$^+$CD25$^+$ regulatory T cells modulate immune tolerance and acute exacerbation on the natural history of chronic hepatitis B virus infection

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Summary

Acute exacerbations (AEs) of chronic hepatitis B (CH-B) are accompanied by increased T cell responses to hepatitis B core and e antigens (HBcAg/HBeAg). Why patients are immunotolerant (IT) to the virus and why AEs occur spontaneously on the immunoactive phase remain unclear. The role of HBcAg-specific CD4$^+$CD25$^+$ regulatory T (T_{reg}) cells in AE and IT phases was investigated in this study. The SYFPEITHI scoring system was employed to predict MHC class II-restricted epitope peptides on HBcAg overlapping with HBeAg that were used for T_{reg}-cell cloning and for the construction of MHC class II tetramers to measure T_{reg} cell frequencies ($T_{reg}f$). The results showed that HBcAg-specific $T_{reg}f$ declined during AE accompanied by increased HBcAg peptide-specific cytotoxic T lymphocyte frequencies. Predominant Foxp3-expressing T_{reg} cell clones were generated from patients on the immune tolerance phase, while the majority of Th1 clones were obtained from patients on the immunoactive phase. T_{reg} cells from liver and peripheral blood of CH-B patients express CD152 and PD1 antigens that exhibit suppression on PBMCs proliferation to HBcAg. These data suggest that HBcAg peptide-specific T_{reg} cells modulate the IT phase, and that their decline may account for the spontaneous AEs on the natural history of chronic hepatitis B virus infection.

Abbreviations: AE – acute exacerbation; ALT – alanine aminotransferase; CH-B – chronic hepatitis B; CRI-p – cytotoxicity response index of the peptide; CTLA-4 – cytotoxic T lymphocyte associated antigen-4; ICCS – intracellular cytokine staining; Foxp3 – forkhead family transcription factor box p3; IT – immunotolerant; LITs – liver-infiltrating T lymphocytes; PD1 – program death receptor 1; Th – T helper cell; T_{reg} – regulatory T cell; Tc*f* – cytotoxic T lymphocyte frequency; $T_{reg}f$ – T_{reg} cell frequency

Introduction

The hepatitis B virus (HBV) is not directly cytopathic, and the immune response of the host appears to mediate the hepatocellular injury and

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subsequent viral clearance [1–3]. Women who are chronic carriers of HBV often infect infants in the perinatal or postnatal periods, whereas intrauterine infection is much less common [4]. Neonates born to HBV carrier mothers may be immunologically tolerant to viral proteins to which they were exposed *in utero* [4–6]. The tolerogenic potential of the hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg) is of particular interest because there is evidence that these antigens represent important "targets" for immune-mediated viral clearance [1–3, 7, 8]. The vast majority of untreated infants born to HBeAg-positive mothers become chronic HBV carriers, the so-called perinatally acquired chronic HBV infection [4, 6].

Staged by the immune response of infected hosts, the natural history of perinatally acquired chronic HBV infection can generally be divided into four stages [3, 5, 9–13]. The first stage (Stage 1) is characterized by the presence of serum HBeAg, active viral replication and high serum levels of HBV DNA, but little or no elevation in serum alanine aminotransferase (ALT) levels and no symptoms of illness. Stage 1 is also referred to as immune tolerance phase. Patients on the following Stage 2 are characterized by fluctuations in serum ALT levels associated with positive serum HBeAg, diminished HBV DNA levels and symptoms of illness of active hepatitis (referred to as HBeAg-positive chronic hepatitis). Stage 2 is also designated immunoactive phase [3] or immune clearance phase [5, 10, 11]. Stages 1 and 2 are grouped as replicative phase. Early Stage 3 may overlap with late Stage 2, clearance of bulk of virus-infected hepatocytes has occurred, mediated by the host immune response, resulting in cessation of viral replication and in the development of anti-HBe antibodies (HBeAg seroconversion) [10–13]. Late Stage 3 may also overlap with early Stage 4, characterized by the decline of immune response accompanied by very low or undetectable serum levels of HBV DNA and normalization of ALT levels. Stage 4 is also referred to as the inactive-carrier state, or "healthy" hepatitis B surface antigen (HBsAg) carrier state [9]. Some Stage 4 patients may clear HBsAg with subsequent appearance of anti-HBs antibodies, especially in patients receiving antiviral treatments [9, 13]. Late Stage 3 and Stage 4 are grouped as nonreplicative phase, which is also referred to as "late integrative phase" or "residual phase" because active replication of the virus ceases and the liver displays only residual lesions resulting from previous insults suffered during the replicative phase of the virus. The HBcAg and HBeAg proteins disappear in association with the integration of HBV genome into the host chromosome. Serum HBV DNA also become barely detectable, even by polymerase chain reaction (PCR) assays [10–13].

Up to date, the reasons why Stage 1 patients are immunotolerant (IT) to the virus, and why hepatitis flares up, i.e. acute exacerbations (AEs), spontaneously in patients on immunoactive phase remain unclear on the natural history of perinatally acquired chronic HBV infection [3, 5, 9–13]. It is shown that AEs of chronic hepatitis B (CH-B) are accompanied by increased T cell responses to HBcAg and HBeAg [14]. Whether HBcAg- and/or HBeAg-specific $CD4^+CD25^+$ regulatory T (T_{reg}) cells modulate AE and IT phases in CH-B patients has not yet been investigated. This study employed the SYFPEITHI scoring system [15] to predict MHC class II-restricted epitope peptides on HBcAg overlapping with HBeAg. The predicted epitope peptides were used for cloning T_{reg} cells and T helper (Th) cells and for the construction of MHC class II tetramers to serially measure HBcAg peptide-specific T_{reg} cell frequencies ($T_{reg}f$) in perinatally acquired chronic HBV patients.

Materials and methods

Study subjects

Fifty-one HBeAg-positive, perinatally acquired chronic HBV patients with HLA-A2 haplotype (28 on the IT phase and 23 on the immunoactive phase) were recruited for this study. They were selected from a screen of 1020 HBV carriers living in Southern Taiwan, Tainan county. All were perinatally acquired chronic HBV infection as they had a clear history of familial HBV infection and with the presence of HBsAg and HBeAg in the serum for at least 6 months. None of them received antiviral treatments before or during the study period. Informed consent was obtained from each study subject and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and approved by the local ethical committee. The demographic data of

the study subjects are listed in Table 1. Of the 23 immunoactive subjects, there are 4 with DRB1*0101 and 3 with DRB1*0401 alleles, respectively.

Patients on AE or acute flare of CH-B is defined as an abrupt elevation of serum ALT level to greater than 5 times the upper limit of normal (ULN = 40 U/l), i.e., ALT > 200 U/l , and without superinfection with any other hepatotropic viruses [11, 16]. Serum HBV DNA was quantified using a hybrid capture assay (Digene HBV test Hybrid Capture II; Digene Corp., Gaithersburg, MD). The detection limit of HBV DNA by this assay is 0.5 pg /ml or 1.4×10^5 copies/ml. 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). Those with HLA-A2 haplotype were subsequently confirmed to have HLA-A2.1 allele by DNA typing (*Micro SSP™, HLA class I DNA Typing Tray-A*02*, One Lambda). DRB1*0101 (DR1) and DRB1*0401(DR4) typing were also conducted with DNA typing (*Micro SSP™, HLA class II DNA Typing Trays DRB1*01 and DRB1*04*, One Lambda). Patients with HLA class II haplotypes other than DR1 and DR4 were included as mutual controls to ensure the specificity of the MHC class II tetramer assay. Because only MHC class II tetramers of DRB1*0101 and DRB1*0401 molecules were commercially available, this study was therefore focused on patients with these two haplotypes.

Table 1. Demographic data of the study subjects.

	Immunoactive group (n = 23)	Immune tolerance group (n = 28)
Age (y/o)	29 (19–57)[a]	22 (17–33)
Sex (M/F)	13/10	(12/16)
ALT (U/l)[b]	135 (51–733)	24 (20–35)
HBV DNA (pg/ml)[c]	301 (122–1779)	507 (163–9842)
DRB1*0101[d]	4	6
DRB1*0401[d]	3	6

[a]Digits represent median (min-max).
[b]Upper limit of normal = 40 U/l.
[c]By a Hybrid Capture assay (Digene Hybrid Capture II HBVDNA Test, Digene Corp., Gaithersburg, MD). The detection limit of HBV DNA is 0.5 pg/ml or 1.4×10^5 copies/ml.
[d]By DNA typing, numbers indicate positive cases.

Preparation of HLA-A2-HBcAg$_{18\text{-}27}$ tetrameric complexes

HLA-A2-restricted epitope peptide HBcAg$_{18\text{-}27}$ FLPSDFFPSV, an immunodominant cytotoxic T lymphocyte (CTL) epitope on HBcAg, is currently the most widely used epitope for assaying CTL activities in HBV patients [2, 17–21]. We used HLA-A2-HBcAg$_{18\text{-}27}$ tetrameric complexes in this study to monitor CTL activities for the correlation with $T_{reg}f$ during AEs in HLA-A2 patients. The tetrameric complexes were prepared as described previously [21].

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

Epitopes on HBcAg restricted by MHC class II antigens, DRB1*0101 and DRB1*0401 molecules have not yet been identified. To ensure the success of tetramer synthesis with 15-mer epitope peptides on HBcAg overlapping with HBeAg, the SYFPEITHI scoring system [15] was utilized to predict the possible epitope peptides that could bind to DRB1*0101 and DRB1*0401 molecules. 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. The 17 peptides with 15-mer amino acids used in this study were arbitrarily designed (their SYFPEITHI scores not shown, Supplementary Table 1), of which, P2, LSFLPSDFFP SVRDL (HBcAg$_{16\text{-}30}$) and P13, PPAYRPPNAPILSTL (HBcAg$_{129\text{-}143}$) had the highest SYFPEITHI scores, i.e., 20 and 28, for DRB1*0401 and DRB1*0101 molecules, respectively. They were used for the construction of each of MHC class II tetramers. All tetramers were purchased from Beckman Coulter, Inc., San Diego, CA (*Kit Lot No. C505190* and *C510066*, respectively). As assay

controls, tetramers of 15-mer peptides derived from HBsAg (P16 and P17, as control of P13 and P2, respectively) were included for the assay.

In vitro short-term culture of liver-infiltrating T lymphocytes with HBcAg peptides

Increasing evidence indicates that short-term culture of PBMCs with the presence of specific antigen may increase the number of antigen-specific T cells and facilitates tetramer staining, flow cytometry cell sorting, and tracking of these T cells during viral infections [22–27]. Culture temperature and activation status of these T cells may also affect the efficiency of tetramer staining [25–27]. Because only small number of liver-infiltrating mononuclear cells, mostly, liver-infiltrating T lymphocytes (LITs), can be isolated from biopsy specimens, we used the modified cytotoxicity response index of the peptide (CRI-p) culture method [22] to expand total cell number of HBcAg peptide-specific T cells and to optimize tetramer staining on LITs from liver biopsy specimens. LITs were collected from fresh liver biopsy specimens of selected patients by removal of hepatocytes using the methods described previously [28]. The culture procedures using the 15-mer peptide P2 or P13 combined with recombinant HBcAg (rHBcAg) to stimulate LITs were carried out as described [20, 22]. Generally, the CRI-p culture method gives 5- to 15-fold increase in total T_{reg} cell numbers compared to that obtained directly from fresh LITs or PBMCs *ex vivo* [20, 22].

$CD4^+CD25^+$ T_{reg} cell depleted PBMCs proliferation assay

$CD4^+CD25^+$ T_{reg} cells were depleted from the PBMCs of seven IT patients by affinity-purified method using Dynal® $CD4^+CD25^+$ T_{reg} kit (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's instructions. Also, PBMCs from another eight IT patients without T_{reg} cell depletion were included as controls for the assay. In 96-well flat-bottomed microculture trays (Nunc, Roskilde, Denmark), T_{reg} cell depleted and undepleted PBMCs were plated at a density of 1×10^5 cells per well in culture medium with the addition of 1 μg/ml rHBcAg and then subjected to a 7-day proliferation assay as described previously [14, 29]. Blocking assays using mouse anti-human program death receptor 1(PD1) (BD Biosciences, Pharmingen) and anti-human cytotoxic T lymphocyte associated antigen-4 (CTLA-4) or CD152 (R & D Systems, Inc.) were performed by coculturing PBMCs with each antibody at a concentration of 10 ng/ml combined with 1 μg/ml rHBcAg.

Limiting-dilution T-cell cloning of HBcAg peptide-specific T_{reg} cells

Total $CD4^+$ T cells were isolated from PBMCs of seven patients on immunoactive phase and three on IT phase with DRB1*0401 or DRB1*0101 (Table 2) using Dynabeads CD4® (Dynal Biotech ASA). These $CD4^+$ T cells from each patient were subjected to limiting dilution T-cell cloning for the generation of $CD4^+$ T cell clones specific to peptide P2 or P13. Briefly, cells were seeded between 25 and 800 cells per well in 96-well, flat-bottomed microculture trays (Nunc, Roskilde) with a dilution factor of two as described [29–32]. Duplicate cloning experiments were carried out for each case. Thus 12 microculture trays (96 replicate culture wells × 12 plates = $1152 \times 2 = 2304$ cloning wells in total) were established for each cloning of each patient. Peptides P2 and P13, each at 1 μg/ml was added to stimulate DRB1*0401 and DRB1*0101 cells, respectively. Mitomycin C (Sigma-Aldrich Co.)-treated autologous PBMCs after T_{reg} cell depletion were used as feeder layer at 10^5 cells per well with addition of recombinant human interleukin-2 (IL-2) (R & D Systems, Inc. Minneapolis, MN) at 20 ng/ml in all cloning wells. Culture medium was refreshed every other day with IL-2 medium at 20 ng/ml. Wells exhibiting cell growth were expanded with IL-2 medium and in turn subjected to a rapid screening of forkhead family transcription factor box p3 (Foxp3)-expressing T_{reg} cell lines with flow cytometry by intracellular cytokine staining (ICCS) method and peptide-specificity analysis. For T-cell lines with equivocal cytokine expression results analyzed by flow cytometry, reverse-transcription polymerase chain reaction (RT-PCR) analysis was further performed for confirmation of the expressions of cytokines and Foxp3 using specific primers (Supplementary Table 2) with amplification conditions as described [29, 33].

Table 2. Patients for T-cell cloning and cytokine expression patterns of HBcAg peptide-specific cloned T-cell lines.

Patient no.	Age (years)	Sex	DRB1 genotype	Phase of T-cell cloning	No. of TCCs	T_{reg} (%)	Th1 (%)	Th2 (%)	*p*-value[c]
1	29	F	0401	AE	30[a]	2 (7%)	28 (93%)	0 (0%)	Overall: $\chi^2 = 358.07$ $p < 0.001$
2	40	M	0401	AE[e]	27[a]	0 (0%)	27 (100%)	0 (0%)	T_{reg} vs. Th1: $\chi^2 = 181.39$ $p < 0.001$
3	55	M	0401	pre-AE	57[a]	4 (7%)	50 (87.7%)	3 (5.3%)	T_{reg} vs. Th2: $\chi^2 = 24.87$ $p < 0.001$
				AE	20[a]	1 (5%)	19 (95%)	0 (0%)	Th1 vs. Th2: $\chi^2 = 393.16$ $p < 0.001$
4	23	M	0101	AE	60[b]	3 (5%)	56 (93.3%)	1 (1.7%)	
5	37	F	0101	AE	72[b]	4 (5.6%)	67 (93%)	1 (1.4%)	
6	43	M	0101	pre-AE	81[b]	12 (14.8%)	67 (82.7%)	2 (2.5%)	
				AE	49[b]	5 (10.2%)	44 (89.8%)	0 (0%)	
7	42	F	0101	pre-AE	55[b]	7 (12.7%)	45 (81.8%)	3 (5.5%)	
				AE	40[b]	2 (5%)	38 (95%)	0 (0%)	
8	18	M	0101	IT	29[b]	9 (31.0%)	2 (6.9%)	18 (62.1%)	
9	23	F	0101	IT	31[b]	11 (35.5%)	2 (6.5%)	18 (58.0%)	
10	23	F	0401	IT[e]	33[a]	12 (36.4%)	0 (0%)	21(64.6%)	

[a]P_2-specific;
[b]P_{13}-specific;
[c]All comparisons were performed by χ^2 test.
[e]Cloned from liver-infiltrating lymphocytes; others from PBMCs.
AE, acute exacerbation; IT, immune tolerance; TCC, T-cell clone.

Flow cytometry analysis and ICCS

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 staining included anti-PD1, anti-CD152, anti-CD4, anti-CD8, anti-CD25 (Immunotech Co, Marseille, France). ICCS study was conducted using mouse anti-human-Foxp3 PE (Biolegend, San Diego, CA), anti-human IL-4-PE (Immunotech Co), IL-12-PE (Biolegend), IFN-γ-FITC (Immunotech Co), and IL-10-PE (Serotec, Oxford, UK). Intracellular TGF-β1 was stained by an indirect immunocytochemical method using a mouse anti-human TGF-β1 monoclonal antibody (hybridoma clone 9016, R&D System, Inc.) as the first antibody and goat anti-mouse IgG as the second antibody (Chemicon International, Temecula, CA).

Statistical analysis

Statistical analysis was performed with the SPSS software version 12.0 (SPSS Inc. Chicago, IL). 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

MHC class II-restricted epitope peptides predicted by the SYFPEITHI scoring system correlated with proliferation assays

Figure 1a shows proliferation assay results of affinity-purified $CD4^+CD25^+$ T_{reg} cells from fresh PBMCs of one DRB1*0101 and non-DRB1*0101patient each on IT phase in response to peptides P13 and P10, and rHBcAg. A significant proliferation of $CD4^+CD25^+$ T_{reg} cells from the DRB1*0101 patient responding to both peptide P13 and rHBcAg was detected, but undetectable proliferation to P10 peptide with an SYFPEITHI score " 0 " on DRB1*0101 molecule (Supplementary Table 1). The proliferation of $CD4^+CD25^+$ T_{reg} cells from the non-DRB1*0101 patient also revealed detectable proliferation to P13 peptide (but much lower than that of DRB1*0101 patient) and P10 peptide (significantly higher than that of DRB1*0101 patient). These data were compatible with the SYFPEITHI score of P13 peptide (= 28

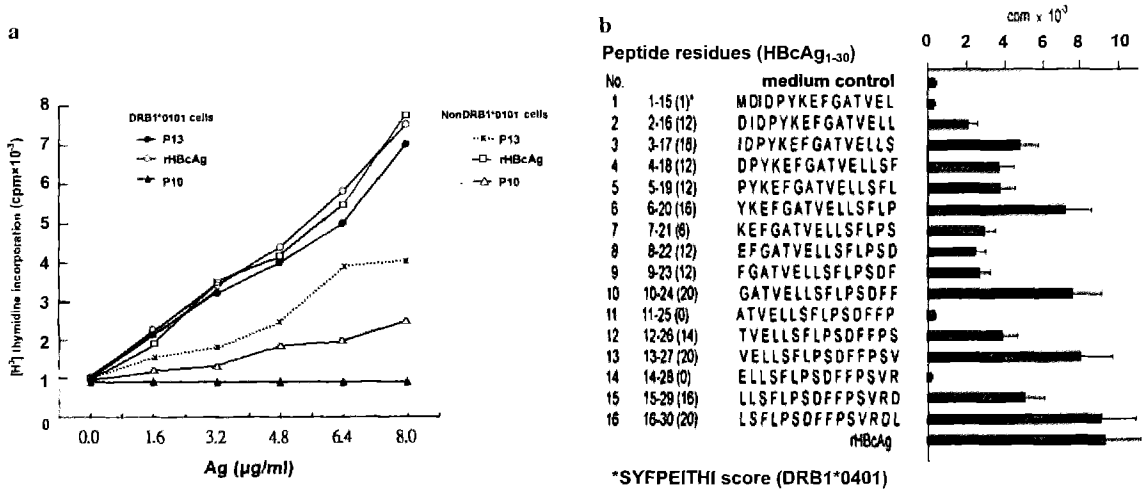

Figure 1. MHC class II-restricted epitopes predicted by the SYFPEITHI scoring system correlated with proliferation assays. (a) Shows results of proliferation assay of affinity-purified $CD4^+CD25^+$ T_{reg} cells from fresh PBMCs of one DRB1*0101 and non-DRB1*0101patient each on IT phase responding to peptides P13 and P10, and rHBcAg. A significant proliferation of $CD4^+CD25^+$ T_{reg} cells from the DRB1*0101 patient to both peptide P13 and rHBcAg was detected, but undetectable proliferation to P10 peptide with an SYFPEITHI score "0" on DRB1*0101 molecule. There was also detectable proliferation of $CD4^+CD25^+$ T_{reg} cells from the non-DRB1*0101 patient to P13 peptide (but much lower than that of DRB1*0101 patient) and P10 peptide (significantly higher than that of DRB1*0101 patient). The prediction of 15-mer peptides of continuous amino acid sequences on $HBcAg_{1-30}$ using the SYFPEITHI scores also correlates proliferation assays in DRB1*0401 patients in terms of $[H^3]$ thymidine incorporation (Figure 1b). Ag, peptides or rHBcAg. cpm, counts per minute.

on DRB1*0101 molecule), consequently P13 peptide can serve as an epitope on HBcAg suitable for the construction of DRB1*0101 tetramer. Likewise, Figure 1b shows the prediction of 15-mer peptides of continuous amino acid sequences on $HBcAg_{1-30}$ using the SYFPEITHI scoring system on DRB1*0401 molecule correlates proliferation assays of $CD4^+CD25^+$ T_{reg} cells from DRB1*0401 patients in terms of $[H^3]$ thymidine incorporation. The proliferation in response to peptide P16 ($HBcAg_{16-30}$, with an SYFPEITHI score = 20 on DRB1*0401 molecule) was comparable to that of rHBcAg, thus this peptide (as P2 in Supplementary Table 1) was selected for the construction of DRB1*0401 tetramer in this study. These data of proliferation are consistent with reports that MHC class II tetramers may identify peptide-specific human $CD4^+$ T cells proliferating to viral antigens [27, 34], and with that T_{reg} cell population could be induced and expanded by foreign antigen [35]. In spite of the facts that anergy and absence of proliferation is considered a specific characteristic of T_{reg} cells [36], while these T_{reg} cells have been long-term priming with HBV antigens and/or HBV-derived peptides processed by virus-infected hepatocytes and other antigen-presenting cells of HBV patients since perinatal period, therefore the detection of antigen-specific proliferation of $CD4^+CD25^+$ T_{reg} cells responding to HBV antigen and/or peptides should not be interpreted as unusual. Moreover, we have demonstrated that these $CD4^+CD25^+$ T_{reg} cells may express IL-2 (data not shown, Supplementary Figure 1), which is considered essential for the survival of mature Foxp3+ regulatory T cells and its signals are critical for the maintenance of T_{reg} cells in the periphery [37], and may likely enhance their proliferation.

HBcAg-specific $T_{reg}f$ declined during AE accompanied by increased HBcAg-specific CTL frequencies

Figure 2 shows one representative case with spontaneous AE revealing a decrease of total $T_{reg}f$ from pre-AE to peak AE phase accompanied by increased $HBcAg_{18-27}$-teramer staining $CD8^+CD25^-$ cytotoxic T lymphocyte (CTL) frequencies (Tc*f*). To ensure the specificity of peptide-MHC class II tetramer staining, patients negative for DRB1*0401 and DRB1*0101 were studied as controls in whom P2- or P13-specific tetramer

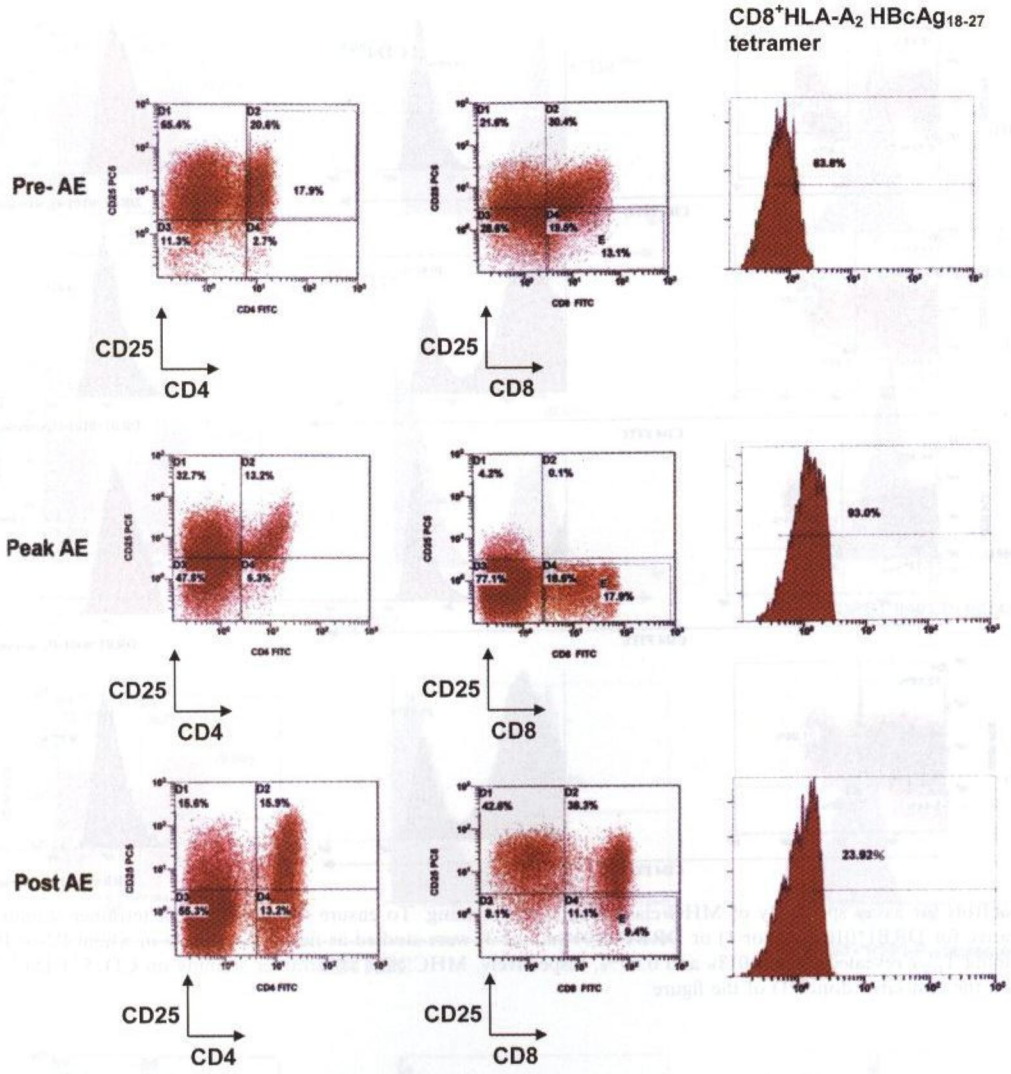

Figure 2. HBcAg-specific $T_{reg}f$ declined during AE accompanied by increased HBcAg-specific CTL frequencies. A representative case of serial follow-up study shows that a decline of total CD^+CD25^+ T_{reg}-cell frequencies from pre-AE to peak AE (left panel) is accompanied by an increase of $HBcAg_{18-27}$-tetramer staining $CD8^+CD25^-$ CTL frequencies (right panel). The data were assayed on PBMCs after short-term *in vitro* modified CRI-p culture. Timing for the assay: pre-AE, two weeks before peak ALT level detected (peak AE); post-AE, two weeks after peak ALT level.

staining $T_{reg}f$ revealed a level below 0.1%, indicating that the tetramer staining was specific for DRB1*0401 and DRB1*0101 T_{reg} cells (Figure 3). Likewise, only trivial or background staining on DRB1*0101 and DRB1*0401 T_{reg} cells was detected by HBsAg peptides P16- and P17-specific tetramers, respectively, indicating that the changes of $T_{reg}f$ were specific for HBcAg peptides (data not shown). Figure 4a, b demonstrate a decline of P2- and P13-specific $T_{reg}f$ in two representative cases with DRB1*0401 and DRB1*0101, respectively. Both cases showed a decline of total frequencies of $CD4^+CD25^+$ T_{reg} cells as well as peptide-MHC class II tetramer staining $T_{reg}f$ from pre-AE phase to AE phase, and increased at post-AE phase subsequently (data of post-AE phase not included in the figures). Figure 5 shows the change patterns of $T_{reg}f$ (Figure 5a) and Tc*f* (Figure 5b) of the follow-up data of 14 episodes of spontaneous AEs in seven immunoactive patients with DRB1*0101 or DRB1*0401. The data of each episode are listed in Table 3.

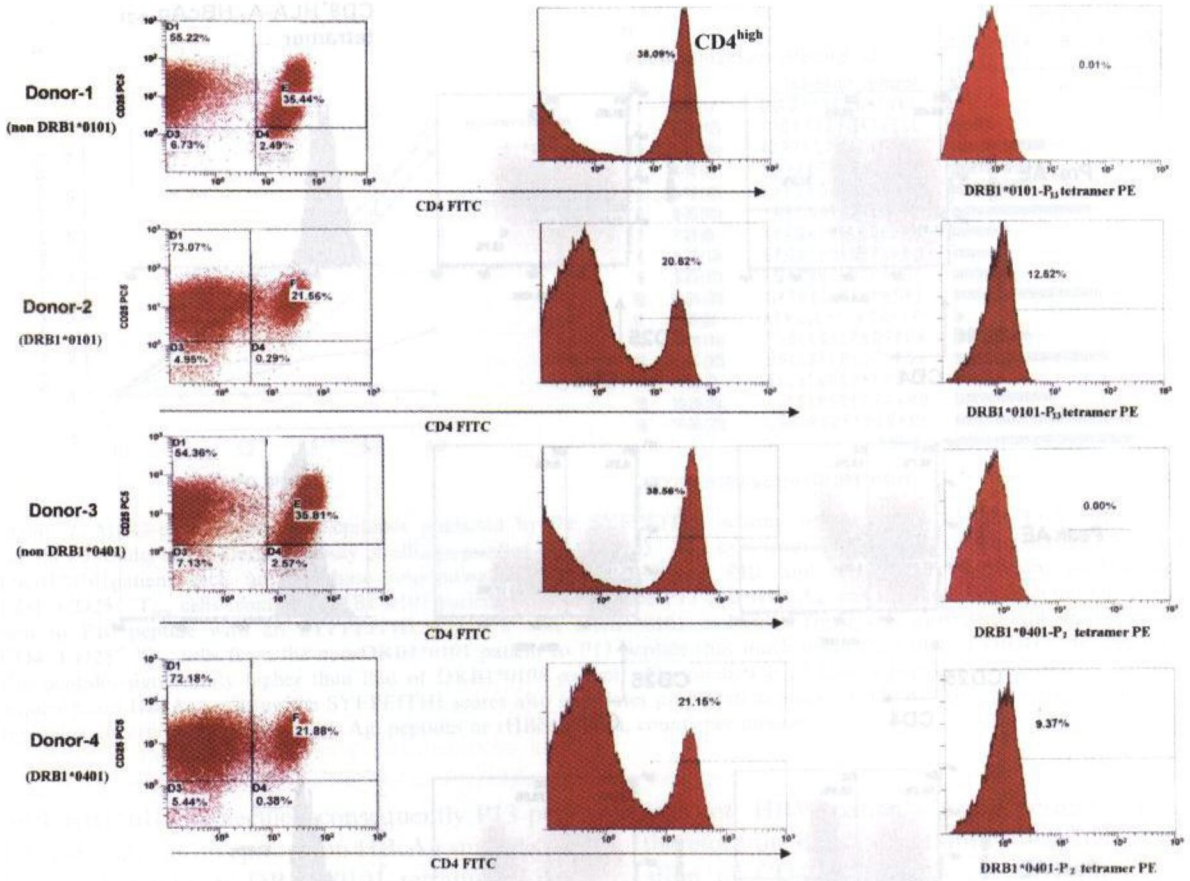

Figure 3. Controls for assay specificity of MHC class II tetramer staining. To ensure the specificity of tetramer staining, 2 CH-B patients negative for DRB1*0101 (donor 1) or DRB1*0401 (donor 3) were studied as negative controls in whom P2-or P13-specific tetramer staining $T_{reg}f$ revealed to be 0.01% and 0.00%, respectively. MHC class II tetramer staining on $CD25^{+}CD4^{-\ high}$ cells is highlighted on the first lane (donor 1) of the figure.

Predominant HBcAg peptide-specific T_{reg} cell and Th2 clones generated from IT patients

By conventional limiting dilution T-cell cloning method, T_{reg} cell clones with secreting TGF-β1 alone (Th3 clone) or IL-10 alone (Tr1 clone) were not identified in this study. Mostly, T_{reg} cell and Th2 clones were generated from IT patients, while the majority of Th1 clones were obtained from immunoactive patients ($p < 0.001$, χ^2 test, Table 2). Identification of these T-cell clones was according to the expression of Foxp3 [38] (data not shown, Supplementary Figure 2), and cytokine expression patterns [39, 40]. Figure 6a shows RT-PCR analysis of T_{reg} cell clone-1 expressing IL-2 (consistent with ICCS study, Supplementary Figure 1), IFN-γ, IL-10, TGF-β1, and Foxp3, as well as both IL-12Rβ1 and IL-12Rβ2, but undetectable IL-4 and IL-12. This result was consistent with the report that T cells producing both IFN-γ and IL-10 in persistent infections are implicated to play an important role in modulating the immune response against intracellular parasites [41]. Figure 6b–d represent one Th1 clone, one Th2 clone and T_{reg} cell clone-2, respectively. Compared with Figure 6a, Figure 6d demonstrates that T_{reg} cell clone-2 expresses IL-4, IL-10 (low), IFN-γ, IL-12Rβ1 and IL-12Rβ2, and Foxp3, but undetectable IL-12 and TGF-β1. These results suggest that Foxp3 expressing T_{reg} cells may potentially secrete a variety of cytokines. However, more data are required to validate this observation.

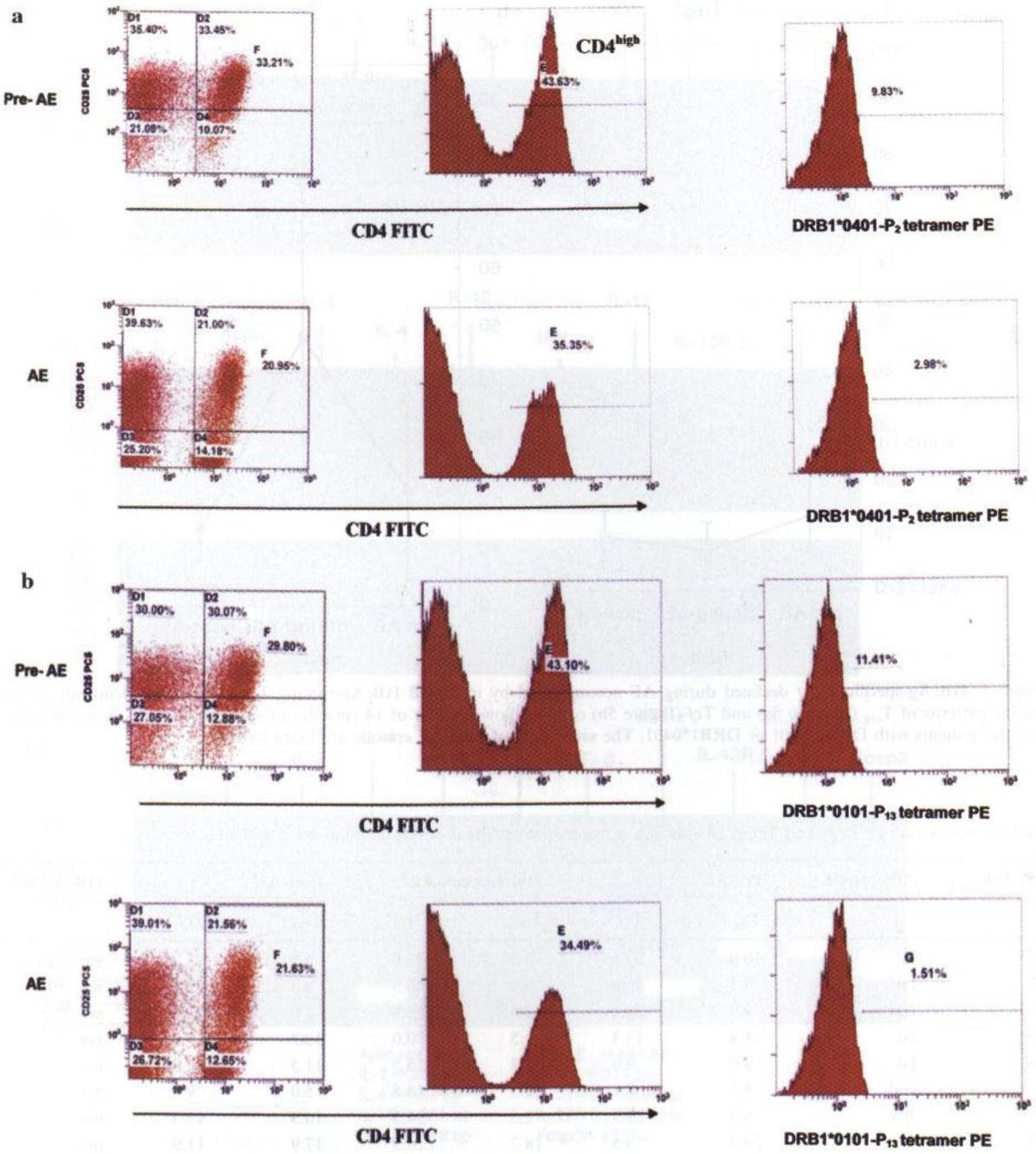

Figure 4. Decline of HBcAg peptide-specific $T_{reg}f$ during AE. A decline of HBcAg peptide-specific, i.e., P2-specific and P13-specific $T_{reg}f$ in two cases with DRB1*0401 (panel a) and DRB1*0101 (panel b), respectively during an AE episode. Three-colored flow cytometry analysis of CD4 and CD25 (left panel), and stained on $CD25^{+}CD4^{+\ high}$ cells with peptide MHC class II tetramers (right panel). The data were obtained by assaying on PBMCs after short-term modified CRI-p culture. In these two cases, pre-AE, three weeks before the peak ALT levels being detected (AE).

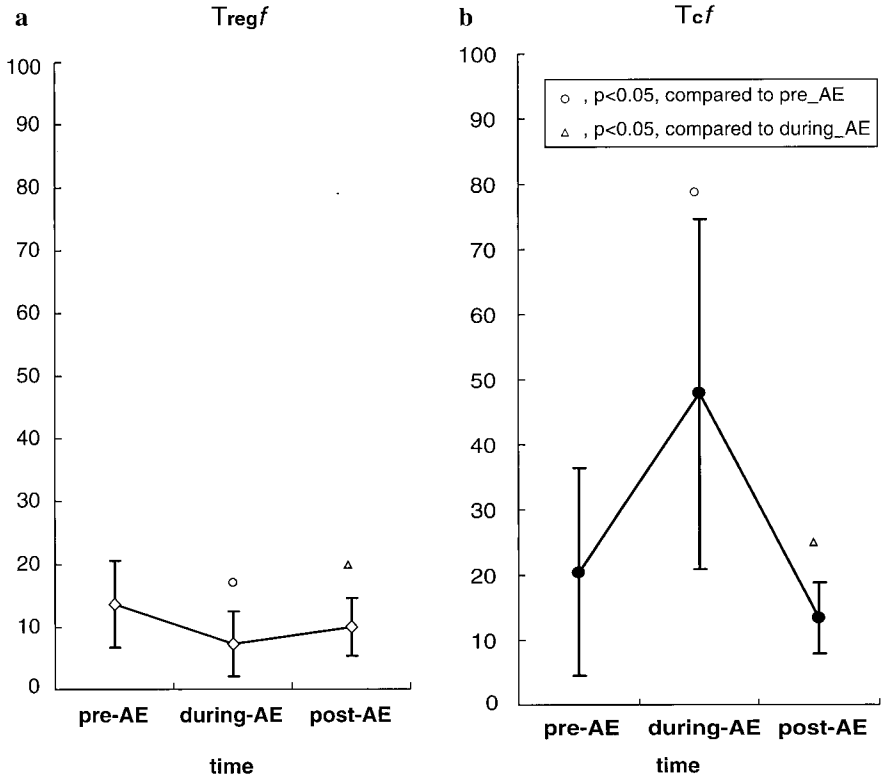

Figure 5. HBcAg-specific $T_{reg}f$ declined during AE accompanied by increased HBcAg-specific Tc*f*. This figure demonstrates the change patterns of $T_{reg}f$ (Figure 5a) and Tc*f* (Figure 5b) of the follow-up data of 14 episodes of spontaneous AEs in seven immunoactive patients with DRB1*0101 or DRB1*0401. The serial data of each AE episode are listed in Table 3.

Table 3. Serial data of $T_{reg}f$ and Tc*f* in 14 episodes of spontaneous AEs in seven immunoactive patients.

Pt No.	AE episode	Pre-AE[a]		During/peak AE[b]		Post-AE[c]		HBeAg-SC[f]
		$T_{reg}f$[d]	Tc*f*[e]	$T_{reg}f$[d]	Tc*f*[e]	$T_{reg}f$[d]	Tc*f*[e]	
1	1st	10.0	63.8	4.2	93.0	4.9	23.9	yes
2	1st	9.5	43.0	9.0	46.5	6.3	22.0	no
	2nd	9.8	25.9	2.9	68.9	7.0	21.9	yes
3	1st	11.4	13.3	1.5	80.0	10.3	10.8	yes
4	1st	8.0	9.0	7.3	15.5	11.5	13.0	no
	2nd	3.1	8.1	2.5	14.5	5.0	9.7	no
	3rd	5.9	23.9	2.5	34.3	10.3	15.5	no
5	1st	20.5	3.5	18.7	20.5	17.9	11.9	no
	2nd	23.0	11.7	8.5	41.9	18.9	12.3	yes
6	1st	24.5	18.5	5.0	66.7	5.9	10.3	no
	2nd	15.9	19.9	2.7	59.5	5.5	6.7	yes
7	1st	11.3	12.4	10.9	15.7	9.9	7.2	no
	2nd	13.5	11.4	14.2	33.4	12.2	13.0	no
	3rd	23.7	21.1	11.8	79.7	13.9	9.8	yes

[a]1–4 weeks before peak AE; [b]Peak ALT level detected; [c]2–6 weeks after the peak AE; [d]By DRB1*0401-P2 tetramer staining T_{reg} cell frequencies ($T_{reg}f$) (Patients 1–3) or DRB1*0101-P13 tetramer staining $T_{reg}f$ (Patients 4–7); [e]By HLA-A2-$HBcAg_{18-27}$ tetramer staining $CD8^+CD25^-$CTL frequencies (Tc*f*); [f]SC, HBeAg seroconverted to anti-HBe.

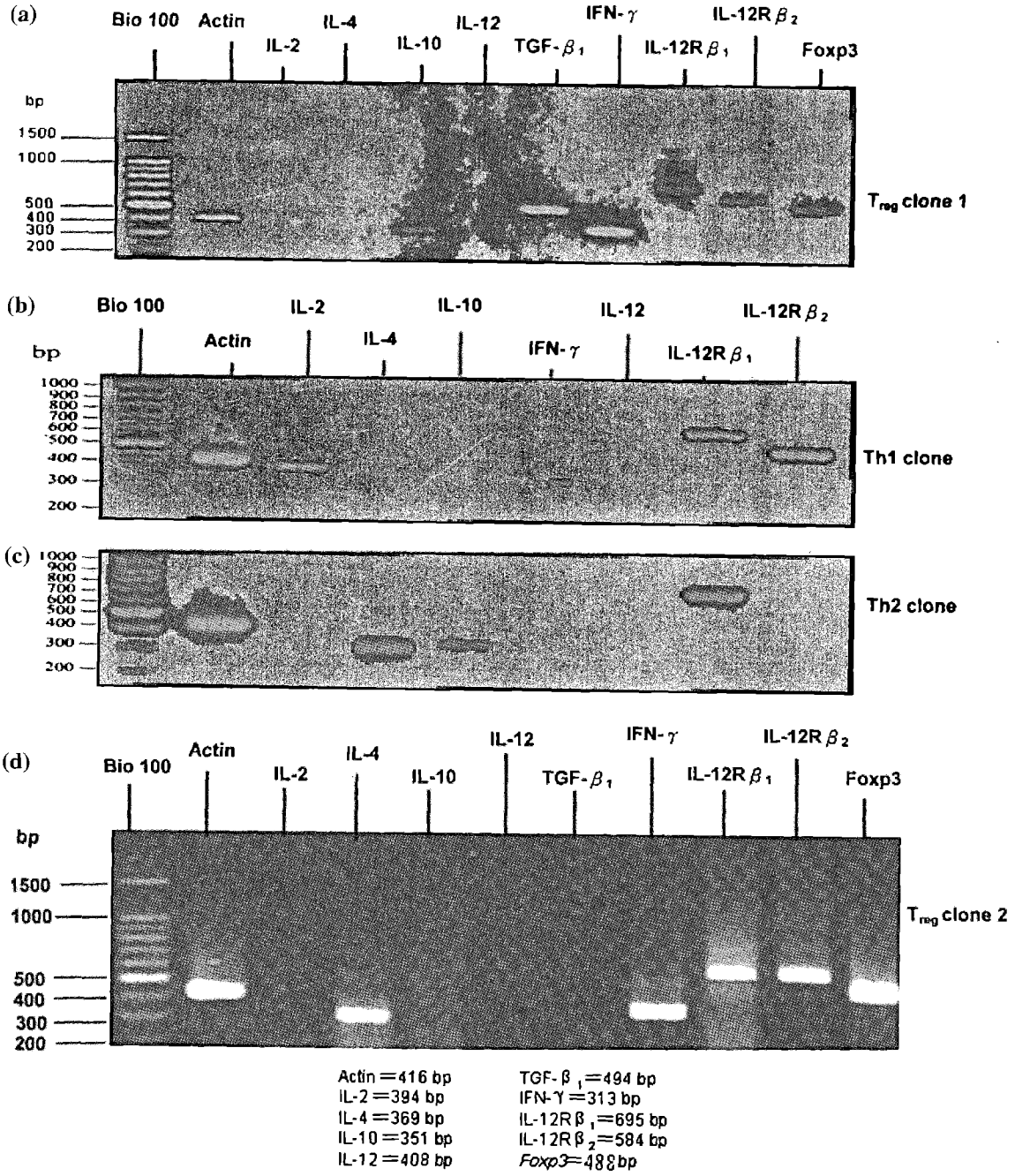

Figure 6. RT-PCR analysis for expressions of cytokines and Foxp3 on cloned T-cell lines. Differentiation among T_{reg} cell clones (a & d), Th1 clone (b) and Th2 clone (c) by RT-PCR analysis of cytokines and Foxp3. It should be noted that both Th1 and Th2 clones do not express Foxp3, and undetectable IL-12Rβ2 expression in Th2 clone.

Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells from HBV patients express PD1 and CD152 (CTLA-4) that exhibit suppression on PBMCs proliferation to rHBcAg

Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells from HBV patients express CD152 and PD1 (data not shown, Supplementary Figures 3 and 4, respectively). They exhibit suppression on PBMCs proliferation to rHBcAg. The depletion of $CD4^+CD25^+$ T_{reg} cells from PBMCs in IT patients significantly enhanced PBMCs proliferation to rHBcAg in terms of increase in stimulation indexes (Figure 7a vs. 7b, $p < 0.001$, Mann–Whitney U test). This enhancement could be reproduced by the addition of anti-CD152 (Figure 7c) and anti-PD1 (Figure 7d) into the cultures, but be abolished by IL-4, IL-10 and TGF-β (data not shown, Supplementary Figure 5). One possible mechanism for the enhanced rHBcAg-stimulated proliferation by anti-CD152 may be via the inhibition of TGF-β secretion which functions as mediator of $D4^+CD25^+$ T suppressor effectors [42]. 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 [43]. The mechanism of our *in vitro* experiments showing the enhancement of PBMCs proliferation to rHBcAg by PD-1 blockade requires further investigation.

Discussion

This study shows that HBcAg-specific $T_{reg}f$ declined during AE accompanied by increased HBcAg-specific cytotoxic T lymphocyte frequencies. The SYFPEITHI scoring system was employed in this study and successfully predicted epitope peptides on HBcAg restricted by DRB1*0401 and DRB1*0101 molecules. Predominant Foxp3-expressing T_{reg} cell clones and Th2 clones were generated from patients on the immune tolerance phase, while the majority of Th1 clones were obtained from patients on the immunoactive phase.

A standard protocol to clone antigen-specific, Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells *in vitro* has yet to be established. Our conventional approach simply used IL-2 and epitope peptides derived from HBcAg as stimulators for the T_{reg} cell cloning. This strategy succeeded unexpectedly in the generation of HBcAg peptide-specific T_{reg} cell clones/lines from PBMCs as well as from liver of CH-B patients despite of low cloning efficiency. Our method to clone T_{reg} cells may be theoretically in agreement with the reports showing that IL-2 is essential for the survival of mature Foxp3+ regulatory T cells and its signals are critical for the maintenance of T_{reg} cells in the periphery [37, 44], and can be consistent with a study showing that T_{reg} cells can be induced and expanded by foreign antigen [35].

Our results may be reconciled with the report from Rossol et al. [45] that Th1 cytokines are important for viral clearance in chronic hepatitis B as predominant Th1 clones were obtained from patients on immunoactive phase. Based on the profiles of T cell clones and/or lines obtained and the data that Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells from HBV patients express PD1 and CD152 exhibiting suppression on PBMCs proliferation to rHBcAg. Our data can also be in agreement with reports that T_{reg} cells may contribute to the impaired immune response to HBV antigens. In patients with chronic HBV infection [46] and possibly, may functionally suppress the activation of $CD8^+$ T cells because our data showed the suppressor effectors' function of Foxp3-expressing $CD4^+CD25^+$ T_{reg} cells [47, 48].

In an HBeAg transgenic (Tg) mice model, in which a function of HBeAg, cross-reacts with HBcAg, may induce immunological tolerance *in utero* [49]. The coexistence of a tolerance to HBcAg and HBeAg T-cell determinants and the production of anti-HBc *in vivo* correlate with the immunological status of neonates born to carrier mothers [4, 6, 50]. The maintenance of T-cell tolerance to HBcAg and HBeAg required the continued presence of the tolerogen, and tolerance persisted for < 16 weeks in the absence of the tolerogens [49, 50]. This model may explain, at least in part, how serum HBeAg maintains Stage 1 patients on the immune tolerance phase [4, 6, 10–13].

The natural history of perinatally acquired chronic HBV infection is punctuated by spontaneous flares or AEs [11, 16]. After AEs, CH-B patients can lose HBV DNA and HBeAg from serum and have a remission. With the subsequent appearance of anti-HBe antibodies, the disease usually evolves from chronic hepatitis to the healthy HBsAg carrier state [9, 11]. Seroconversion

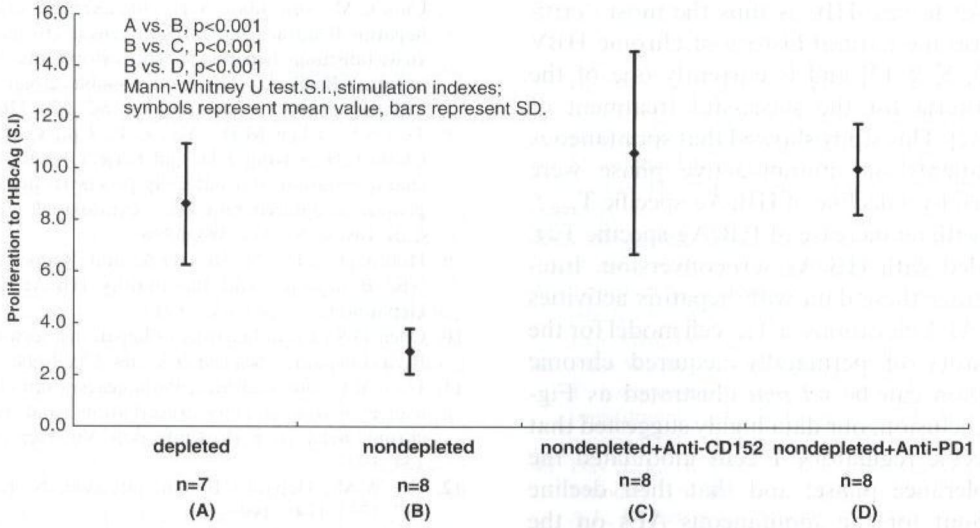

Figure 7. $CD4^+CD25^+$ T_{reg} cell depletion enhanced PBMCs proliferation to rHBcAg. Of the 28 IT patients, 7 cases with $CD4^+CD25^+$ T_{reg} cell depletion, and 8 cases without depletion were studied for proliferation to rHBcAg. For methods of blocking assays on the 8 cases without depletion using anti-PD1 and anti-CD152 antibodies, see text.

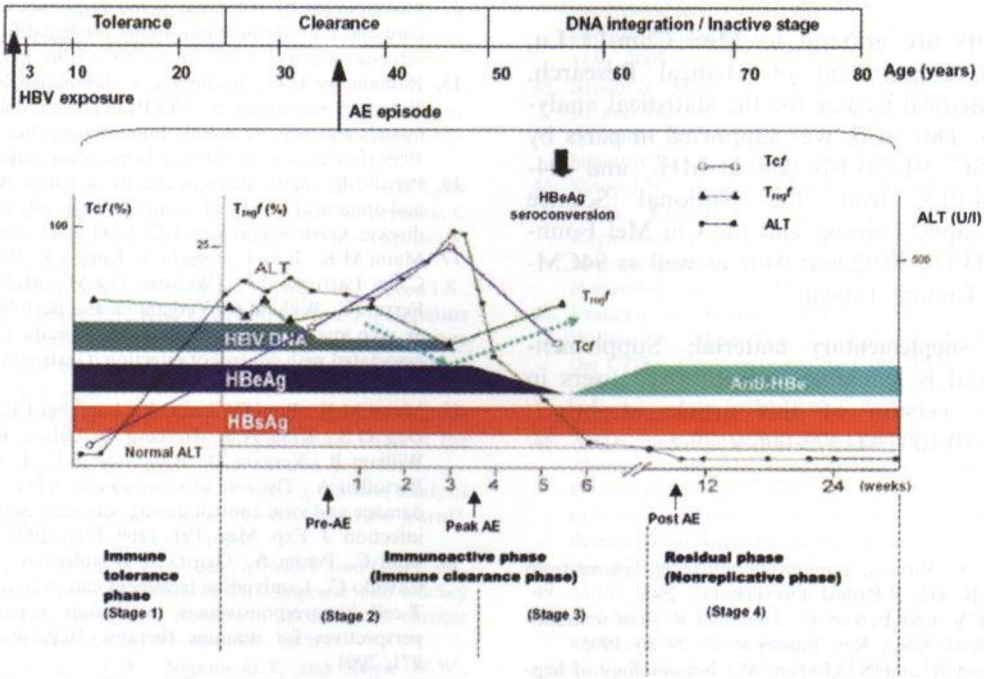

Figure 8. A model of natural history of perinatally acquired chronic HBV infection according to serial changes of $T_{reg}f$ and Tc*f*. The changes of $T_{reg}f$ and Tc*f* in relationship with serum levels of HBV DNA, ALT, and HBeAg start from perinatal period (immune tolerance phase, Stage 1) with normal ALT, low Tc*f*, relative high $T_{reg}f$, high HBVDNA, and positive HBeAg. Stage 2 patients may develop AE spontaneously during immunoactive phase (Stage 2 and early Stage 3, 20–40 years old in age usually). One episode of AE occurred in patients at age around 35 years old is shown here with abrupt elevation of ALT, high Tc*f*, decline in $T_{reg}f$, decreasing HBV DNA, and loss of HBeAg with generation of anti-HBe antibodies (HBeAg seroconversion without the development of pre-core mutant hepatitis). Subsequently patients may have a clinical remission and evolve to an inactive HBsAg carrier state (late Stage 3 and Stage 4, or residual phase). Light green dashed line highlights the changing trend of $T_{reg}f$ during the AE episode.The term "clearance" is kept in the figure [5, 10, 11], but not replaced by "immunoactive" [3] as in the text.

from HBeAg to anti-HBe is thus the most "critical" event on the natural history of chronic HBV infection [3, 5, 9–13] and is currently one of the essential criteria for the successful treatment of CH-B [51, 52]. This study showed that spontaneous AEs of patients on immunoactive phase were accompanied by a decline of HBcAg-specific $T_{reg}f$, associated with an increase of HBcAg-specific Tc*f*, and coincided with HBeAg seroconversion. Integrated together these data with hepatitis activities in terms of ALT elevations, a T_{reg} cell model for the natural history of perinatally acquired chronic HBV infection can be *ad rem* illustrated as Figure 8. In conclusion, our data highly suggested that HBcAg-specific regulatory T cells modulated the immune tolerance phase, and that their decline might account for the spontaneous AEs on the natural history of perinatally acquired chronic HBV infection.

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