Modulation of the CD8+ T cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B infection.

Running title: Natural regulatory T cells in HBV infection.

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Abstract

CD4+ CD25+ regulatory T cells have been shown to maintain peripheral tolerance against self and foreign antigens. In this study we analyse the effect of circulating CD4+ CD25+ T cells on CD8+ T cell response of patients with chronic and resolved HBV infection. We demonstrated that circulating CD4+ CD25+ T cells modulate the function and expansion of HBV-specific CD8+ cells ex vivo in all patients, regardless of whether they have chronic or resolved HBV infection. The possible role of CD4+CD25+ T cells in the pathogenesis of chronic HBV infection is not supported by these data. However, these results might have implications for optimising future immunotherapeutic approaches to HBV treatment.
**Introduction**

HBV is a non-cytopathic, hepatotropic DNA virus that infects more than 300 million people worldwide, causing liver disease of variable severity (15). The pathogenesis of the liver damage during HBV infection is immune-mediated, and is dependent on the balance between viral replication and the CD8+ T cell response (7). Virus-specific CD8+ cells are necessary for HBV control (25) but are defective in patients with persistent HBV infection when compared with those who resolved infection (11). High antigen dose deletion (28) and lack of CD4 help (13) could explain the low number of virus-specific CD8+ cells present in patients with chronic infection. However, it remains possible that other direct mechanisms of regulation of CD8+ expansion operate in patients with chronic HBV infection, that still possess low frequencies of virus-specific CD8+ cells in lymph nodes (18), liver (17) and blood (28). The suggestion that a residual population of cells is actively suppressed in chronic HBV infection is supported by the boosting of their frequencies on reduction of viral load with antiviral therapy (9).

Studies in a large number of experimental models have provided convincing evidence that a population of specialised T cells able to actively regulate the immune response represents an integral part of the T cell repertoire (19, 21). These cells have been shown to suppress immunological responses against self (3, 22) and foreign antigens (1, 6, 12, 24) and reside mainly, but not exclusively, within a minor subpopulation of CD4+ cells that express the phenotypic marker CD25 (4). The mechanisms that mediate the regulatory effect of CD4+ CD25+ cells are still controversial, with evidence supporting regulation either through suppressive cytokines or direct cell-cell
CD4+ CD25+ regulatory cells develop in the thymus and are anergic to antigenic stimulation in vitro, but recent experiments have demonstrated their ability to expand \textit{in vivo} following Ag recognition(27). This ability of CD4+ CD25+ to respond to peripheral antigens could be particularly important in the regulation of immunopathological and protective responses to parasites (6) and viruses (1, 6, 12, 24). In mice infected with HSV, CD4+ CD25+ cells were not only shown to regulate the clonal expansion of virus-specific CD8+ cells, but their suppressive function was also enhanced by the virus infection (24). These data suggest that during viral infection, CD4+ CD25+ cells can be modulated in the periphery after recognition of viral antigens. Furthermore, recent data in HCV-infected subjects has shown the potential ability of CD4+ CD25+ to regulate HCV-specific T cells in patients with chronic hepatitis C(23). These data raise the possibility that a dynamic regulation of virus-specific CD8+ responses can be mediated by CD4+ CD25+ during viral infection.

It is possible that CD4+ CD25+ cells are activated \textit{in vivo} to suppress the expansion of the HBV-specific CD8+ cells able to escape deletion, thus precluding HBV clearance, but limiting excessive immune-mediated liver damage. To test this possibility, we explored the impact of circulating CD4+ CD25+ regulatory T cells in patients with chronic and resolved hepatitis B. We investigated whether in vivo frequencies of CD4+ CD25+ cells differ according to the clinical outcome of HBV infection or correlate with the fluctuation of disease activity present during chronic infection. The direct influence of CD4+ CD25+ cells on the expansion and function of HBV-specific CD8+ cells from patients with chronic and resolved infection was then examined in vitro.
Material and methods

Patients

Blood was collected with informed consent from 40 patients infected with HBV. The study was approved by the local ethics committee. Three subjects (R1, R2, R3) had clinical, biochemical and virological evidence of resolved acute hepatitis B infection (recovered from acute hepatitis B: normal ALT levels, anti-HBc+, HBsAg negative). The remaining 37 patients had clinical, biochemical and virological evidence of chronic HBV infection. They were HBsAg and anti-HBc positive and negative for antibodies to hepatitis C virus (HCV), delta virus and to HIV-1, 2. Patients were not treated with antiviral therapy in the preceding 6 months, and had no other possible aetiologies for chronic liver diseases, such as alcohol, drugs, congestive cardiac failure or autoimmune disease. Frequency of CD4+ CD25+ cells was also tested in HBeAg+ patients who display drug-induced episodes of hepatic flares. These patients received, after the initial screening, 4 weeks of prednisolone treatment (30mg/day for 2 weeks and 15mg/day for consecutive 2 weeks, followed by 2 weeks without treatment) and at week 6 they started Lamivudine (100mg/day). Hepatic flares occur when patients were on Lamivudine treatment only and 3-4 weeks after the discontinuation of prednisolone. The frequency of CD4+ CD25+ T cells was analysed before, during and after episodes of hepatic flares, when patients were on Lamivudine treatment. Blood from healthy donors who were negative for any serological markers of past or present HBV infection was also collected.
PBMC from HBV infected patients and healthy donors were isolated from heparinized whole blood by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Cells were washed twice with RPMI 1640 (Autogen Bioclear UK Ltd, Calne, UK) and suspended in RPMI 1640 + 5% AB serum for further analysis. Screening for HLA-A2 positivity was performed by staining PBMC of patients with fluorescent conjugated anti-HLA-A0201 antibody (Serotec Ltd, Oxford, UK).

**Virological Assessment.** HBsAg, anti-HBs, total and IgM anti-HBc, HBeAg, anti-HBe, anti-delta, anti-HCV, anti-HIV-1 and anti-HIV-2 were determined by commercial enzyme immunoassay kits (Abbot Laboratories, North Chicago, IL; Ortho Diagnostic System, Raritan, NJ; Sanofi Diagnostic Pasteur, Marnes-la Coquette, France). Serum HBV-DNA was quantified by using the Roche Amplicor Monitor assay (Roche Pharmaceuticals Ltd., Branchburg, NJ), with a DNA detection limit threshold of 400 copies/ml (0.0014 pg/ml).

**Synthetic peptides.** Peptides corresponding to the sequence of HBV genotype D were purchased from Primm (Milano, Italy). Purity was above 90% using HPLC analysis. The AA sequence of the peptides used is: core 18-27 = FLPSDFFPSV, env 183-91 = FLLTRILTI, env 348-57 = GLSPTVWLSV, pol 455-63 = GLSRYVARL. A melanoma peptide (Melan-A 26-35 = ELAGIGILTV) was used as a control.

**Phenotype of CD4+ CD25+ T cells.** Fresh or frozen PBMC were washed once in phosphate-buffer saline (PBS) containing 1% FCS and stained with fluorescently labelled antibodies for CD4-fluorescein isothiocynate (FITC), CD25-phycoerythin
(PE) or anti-IgG2 control antibody (BD Biociences PharMingen, San Diego, California) for 20 min at 4°C. The cells were then washed twice with PBS containing 1% FCS, and immediately acquired on a flow cytometer FACScan™ (Beckton Dickinson) and analysed using Cell Quest software. Frequency of CD4+CD25+ cells was analysed in frozen PBMC with viability > 80% (by Trypan Blu analysis). Experiments showed that within this viability range, the CD4+ CD25+ T cell frequency was identical in fresh or frozen samples.

**Depletion of CD4+ CD25+ cells.** CD4+ CD25+ cells were isolated from PBMC using the CD4+ CD25+ Regulatory T cell isolation Kit (Myltenyi Biotec, Auburn, CA), with a Midi Macs™ Separator unit, according to the manufacturer instructions. The efficiency of CD4+ CD25+ T cell depletion was > 90%. Untreated PBMC or PBMC depleted of CD4+ CD25+ cells were then used for producing short term T cell lines. In selected experiments, the positively selected CD4+ CD25+ cells were added back to depleted PBMC.

**Expansion of CD8+ T cell lines.** Total PBMC or PBMC depleted of CD4+ CD25+ cells (2-3 x10^6/well) were stimulated in RPMI + 10% FCS with 1µM of various peptides in a U bottom 96 well plate. Recombinant IL-2 (R&D systems, Abingdon, UK) was added on day 4 of culture and frequency and function of CD8+ cells were analysed after 10-12 days of culture. In selected experiments CD4+ CD25+ cells were added back to CD4+ CD25+ depleted PBMC at a responder/regulator ratio of 20:1.

**Intracellular Interferon (IFN)-γ production.** Short-term T cell lines were stimulated in RPMI 1640 10% FCS, with the initial stimulatory peptides (1µM), for 6
hours at 37°C in the presence of 10 µg Brefeldin A (Sigma-Aldrich, Poole, Dorset). Cells were washed, stained with Cy-chrome conjugated anti-CD8 antibodies, then permeabilized and fixed using Cytofix/Cytoperm (Pharmingen, San Diego) according to the manufacturers instructions. FITC-conjugated anti-cytokine antibodies or isotype-matched controls were added (30min, 4°C), washed twice and cells were then analysed by flow cytometry.

**Staining with HLA-tetrameric complexes.** HLA-class I tetramers were purchased commercially (Proimmune, Oxford, UK). Tc18-27 is an HLA-A2 tetramer specific for HLA-A2 restricted core18-27 specific CD8 cells, T mel, is specific for HLA-A2 restricted Mage-1-specific CD8+ cells. The tetramers were used to stain short term lines produced from PBMC of the patients. T cell lines were incubated for 30 min at 37°C with 1µg of phycoerythrin-labelled tetrameric complex in RPMI 1640, 10% FCS in round-bottom polystyrene tubes (Becton Dickinson, NJ). Cells were washed in PBS and then incubated at 4°C for 30 min with saturating concentrations of directly conjugated anti-CD8/Cy-chrome (PE-Cy5) monoclonal antibody (mAb) (Sigma Chemical Co., St. Louis, MO)After further washing, cells were analysed on FACS® (Becton Dickinson) using CELLQuest™ software immediately or after addition of 1% paraformaldehyde.
Results and Discussion

CD4+ CD25+ cells in the circulation of HBeAg+ patients with chronic hepatitis B.

We tested the hypothesis that the inability to control viral replication and the absence of immune-mediated liver damage present in HBeAg+ immunotolerant patients were attributable to high levels of regulatory cells. The proportion of CD4+ CD25+ cells was quantified cross-sectionally in different categories of patients with chronic HBV infection. 15 ‘immunotolerant’ patients (HBeAg+, HBV-DNA>10^7, transaminase levels < 70 U/L), 11 patients with clinical evidence of chronic active hepatitis B (HBeAg+, HBV-DNA>10^7, transaminase levels > 70 U/L) and 11 patients with asymptomatic HBV infection (HBsAg+, HBV-DNA < 10^6, transaminase levels normal) were selected. The frequency of CD4+ CD25+ cells present in these three groups of patients with chronic hepatitis B and a group of 14 healthy controls (Figure 1a) was calculated using the gate shown in Figure 1a, which only selected CD25 high cells (Intensity of fluorescence> 3x10). CD25 high cells, which exhibit regulatory cell function, have been shown to represent about 1-3% of the total CD4+ cell population (4), whereas CD25 low cells can represent up to 15% of CD4+ T cells in healthy subjects. There is variability in the frequency of CD4+ CD25 high T cells within the groups of patients and healthy controls (Figure 1b). However, the mean frequency was 2.4 ± 1.1 in ‘immunotolerant patients, 2.8 ± 1.7 in patients with chronic active hepatitis and 1.9 ± 0.9 in the asymptomatic patients: no statistically significant differences were found between the patients groups and healthy controls (2.5 ± 1.1) (Mann-Whitney test: p<0.73, p<0.71, p<0.08 respectively).
We then explored whether there was any reduction in CD4+ CD25+ cells temporally associated with hepatic flares of chronic hepatitis B. “Flares” of chronic hepatitis are generally interpreted as an indicator of recovery of HBV-specific immunity, which occurs spontaneously or during antiviral treatment (15). Here we quantified the frequency of CD4+CD25+ cells before, during and after episodes of hepatic flares occurring in HBeAg+ immunotolerant patients who have been treated with prednisolone and Lamivudine (see Material and methods). These hepatic flares occurred when patients were on Lamivudine treatment only, 3-4 weeks after the discontinuation of prednisolone. This is a highly specific treatment regime which is likely to influence the pathogenesis of hepatic flares. It does, however, allow us to predict the occurrence of hepatic flares and thus to study immunological events that occur before and during ALT elevation. Figure 1c shows the direct ex vivo frequency of CD4+ CD25+ T cells in 5 patients who displayed elevated ALT (> 100 U/L and twice the initial baseline value). A minimal decrease of CD4+ CD25+ frequency at the time of ALT elevation was found in 3 of these patients (3/5). Only one (1/5) showed a decrease of more than 1% (from 2.6% to 1.4%).

To investigate whether these decreases in circulating CD4+ CD25+ frequencies had any impact on HBV-specific CD8+ responsiveness, we analysed in parallel HBV-specific CD8 and CD4+ CD25+ frequency in two HLA-A2+ patients (Table I). Both of these patients exhibited ALT elevations and fluctuations in CD4+ CD25+ cell frequencies (Figure 2). The frequency of HBV-specific CD8+ T cells was quantified directly ex vivo and after in vitro stimulation. In line with our previous work, HBV-specific CD8+ cells could only be quantified in HBeAg+ patients after a round of in vitro expansion and not directly ex vivo. Of the 11 common HLA-A2 restricted CD8
epitopes tested(28), only env 183-91 and env 348-57 elicited a CD8+ T cell response in patient 3 and env 335-43 elicited a response in patient 7. As shown in figure 2, the fluctuations of HBV-specific CD8+ responses found in these two patients did not unequivocally correlate with increased or decreased frequencies of CD4+ CD25+ cells.

Thus, frequency of circulating CD4+ CD25+ T cells did not correlate with the clinical, virological or immunological parameters present in patients with chronic hepatitis B. Larger groups of patients with different profiles of chronic hepatitis B will need to be analysed to confirm these exploratory data, which are similar to those found in HIV (1) and multiple sclerosis(5). In these two systems, despite identical direct ex vivo frequencies in healthy subjects and patients, CD4+ CD25+ cells showed differential immunoregulatory activity according to clinical outcome. We therefore tested whether circulating CD4+ CD25+ cells found in chronic hepatitis B patients are functionally capable of suppressing immune responses to HBV ex vivo.

**CD4+ CD25+ T cells regulate CD8+ T cell responses ex vivo.** PBMC from chronic hepatitis B patients with or without the CD4+ CD25+ fraction were stimulated with selected peptides corresponding to known HLA-A2 restricted HBV epitopes. The frequency of HBV-specific CD8+ cells was calculated by intracellular IFN-gamma production after 10 days of in vitro expansion (Figure 3).

It is noteworthy that the patients analysed were carefully selected from a larger group of HLA-A2+ patients in whom the profile of HBV-specific CD8+ T cell response had been studied for more than 1 year(28). We selected 4 patients demonstrating the
presence of HLA-A2-restricted HBV-specific CD8+ T cells (Patient 1,2,3,4) and 2 patients with no detectable HLA-A2 restricted HBV-specific CD8+ cells (Patient 5, 6). The clinical, virological and histological data of the selected patients are shown in Table I. Depletion of CD4+ CD25+ cells did not have any impact on HBeAg+ patients with consistently undetectable HBV-specific CD8+ cells for more than 1 year of analysis (Figure 3: Patients 5, 6), in whom such cells are likely to be deleted. In contrast, the frequency of HBV-specific CD8+ T cells was increased in PBMC depleted of CD4+CD25+ cells compared to total PBMC in all the other patients tested, irrespective of their HBeAg or viral replication status. Analysis of specific CD8+ expansion with HLA-tetramers (Tc18-27 and T-Melan-A) elicited results similar to those obtained by ICS (not shown). Importantly, when CD4+ CD25+ cells were added back to the culture, the expansion of HBV-specific CD8+ cells reduced to the levels observed before depletion (Figure 3c). Depletion of the CD4+ CD25+ population affected not only the expansion of HBV-specific CD8+ cells, but also their function. The proportion of tetramer+ CD8 cells able to produce interferon-gamma increased in cells expanded in the absence of CD4+ CD25+ cells (Figure 4).

These results confirm the ability of circulating CD4+ CD25+ to suppress anti-viral immune response mediated by CD8+ T cells (10, 20). However this regulation is clearly non-antigen specific. In parallel with the expansion of HBV-specific CD8+ T cells, we performed control experiments using a Melan-A peptide, an HLA-A2 restricted epitope able to induce CD8+ T cell responses in healthy control HLA-A2+ subjects (26). The impact of CD4-CD25+ depletion on the expansion of Melan-A specific CD8+ T cells was, in many patients, similar to that found in HBV-specific CD8+ cells (Figure 3b).
CD4+ CD25+ regulatory cells in patients with resolved HBV infection. We analysed whether CD4+ CD25+ regulatory cells have an altered suppressive capacity in chronic compared to resolved patients. Differences in the functional suppression mediated by CD4+ CD25+ T cells have been shown in patients with multiple sclerosis versus healthy individuals(5). We therefore analysed whether circulating CD4+ CD25+ cells present in patients with resolved HBV infection can regulate CD8+ T cell expansion. The direct ex vivo frequency of CD4+ CD25+ cells in these patients was similar to that found in healthy subjects (R1=2.4%; R2= 1.7%, R3= 2.8%). As shown in figure 5, depletion of CD4+ CD25+ cells further augmented the already robust HBV-specific CD8+ expansion present in resolved patients, showing that CD4+ CD25+ cells can also regulate CD8+ T cell responses in this setting.

In conclusion, we have analysed the role of circulating CD4+ CD25+ cells in patients with different profiles of HBV infection. We could not find any marked elevation in circulating CD4+ CD25+ in ‘immunotolerant’ HBeAg+ patients, nor any temporal relationship between therapeutically induced disease activity and CD4+ CD25+ cell frequency, even though the pathogenesis of therapeutically induced hepatic flares is likely to differ from those occurring spontaneously.

In addition, the data did not demonstrate a functional difference between the circulating CD4+CD25+ regulatory cells of chronic and resolved patients. Unlike murine HSV(24) and human HCV (23) infection, chronic HBV infection does not appear to be associated with a marked induction of this suppressor subset. However, this study was limited by analysis of the circulating compartment only, and a detailed study of the frequency and function of intrahepatic CD4+CD25+ T cells might reveal
important differences between the heterogeneous populations of chronic patients. It cannot be excluded that antigen-specific regulatory cells (8) are preferentially induced in chronic HBV patients analogous to the presence of IL-10 producing HCV-specific CD4+(16) or CD8+ cells (2) in HCV infected patients. Studies have reported T cell production of IL-10 after stimulation with HBcAg (14).

This study does however demonstrate that circulating CD4+ CD25+ cells are able to functionally suppress activation of CD8+ T cells specific for HBV and for unrelated antigens \textit{ex vivo}. They are able to “fine tune” HBV-specific CD8 responses in the setting of both chronic and resolved infection. This ability to regulate the equilibrium between the impact of the infection and the host response could prevent an excessive pathogenetic response in chronic HBV, and could conversely inhibit complete viral clearance in patients who have resolved HBV, as has been shown in \textit{Leishmania} infection (5). The small increases in HBV-specific CD8 responses seen in patients with chronic HBV on depletion of the CD4+ CD25+ population could actually represent a useful increment to the critically low numbers found in this group of patients and may have practical implications for the clinical management of this subgroup. Future strategies could attempt to down-modulate the number or function of CD4+ CD25+ cells, in order to boost the frequency of HBV-specific CD8 responses prior to therapeutic vaccination.
Acknowledgements

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References


Figure legends.

Figure 1. **Direct ex vivo frequency of circulating CD4+ CD25+ cells in HBV infected subjects.** 1a) PBMC stained with anti-CD4 FITC and anti-CD25 PE. Cells were gated on live lymphocytes on the basis of their forward and side scatter properties. Fluorescent quadrants were set using anti-PE isotype matched control antibody. The frequencies of CD4+ CD25+ high cells were calculated using the quantity of cells included in the indicated (R2) gate. 1b) Direct ex vivo frequencies of CD4+CD25+ high cells out of total CD4 cells in hepatitis B chronic patients and healthy controls. The level of HBV-DNA ( copies/ml) and ALT (U/L) is indicated for each category. 1c) Ex vivo frequencies of CD4+CD25+ high was calculated in the indicated patients 2 weeks before, during and 2 weeks after an episode of therapeutically induced hepatic flare (ALT >100U/L and twice their initial baseline value). These patients were HBeAg+ and on Lamivudine treatment (HBV-DNA 10^5 copies/ml).

Figure 2. **Temporal relationship between CD4+ CD25+ frequency and HBV-specific CD8+ T cell response.** Direct ex vivo frequencies of circulating CD4+ CD25+ cells were calculated at the indicated time points in the two HLA-A2+ patients indicated. In parallel, HBV-specific CD8+ T cell responses were calculated by stimulating PBMC with 10 HBV peptides representing known HLA-A2 restricted epitopes. The frequency of HBV-specific CD8+ cells was calculated with ICS after 8 days of in vitro stimulation. Frequencies of IFN-gamma producing CD8+ cells are
only indicated for responding peptides. Patients were under Lamivudine therapy: HBV-DNA levels was $< 10^5$ copies/ml at all time points.

Figure 3. **Depletion of CD4+ CD25+ cells enhances the expansion of CD8+ T cells.**

All experiments were performed on short term lines derived from PBMC or CD4+ CD25+ depleted PBMC stimulated with HBV and Melan-A peptides. The frequency of peptide specific IFN-gamma producing CD8+ cells was tested with ICS after 8 days of in vitro expansion. 3a) Dot plots show the frequency of env183-91 specific CD8 cells obtained in the different expansion conditions (PBMC or CD4+ CD25+ depleted PBMC). IFN-gamma producing CD8+ cells specific for env 183-91 were quantified with ICS by stimulating cells for 6 h with env 183-91 peptide (+pept). Negative controls are cells not stimulated with peptide (no pept). 3b) Panels indicate the frequencies of IFN-gamma producing CD8+ T cells specific for HBV or Melan-A peptides and obtained in the indicated patients. Note: the scale on the y-axis differs in the panels. 3c) CD4+CD25+ cells regulate CD8 expansion. Purified CD4+ CD25+ were added back into PBMC of Pt1 depleted of CD25+CD4+ cells (PBMC/CD4+CD25+ =20/1). Cells were stimulated with peptide core18-27. The frequency of core18-27 CD8 was analysed after 10 day of in vitro expansion. Bars indicate the frequency of expanded core18-27 CD8+ cells out of total CD8+ cells calculated by staining cells with Tc18-27 tetramers and anti-CD8. The experiment was repeated twice in Patient 1 with similar results.

Figure 4. **Depletion of CD4+CD25+ cells enhances anti-viral function of HBV-specific CD8+ cells.** Visualisation of IFN-gamma production of tetramer+ CD8 cells expanded in the presence or absence of CD4+CD25+. Dot plots represent live gated
CD8+ cells stained with Tc18-27 and anti IFN-gamma antibodies. Numbers indicate the proportion of Tetramer+ CD8+ cells producing IFN-gamma. Results were confirmed in 3 out of 3 different subjects tested.

Figure 5. **CD4+ CD25+ cells regulate the expansion of CD8+ T cells in patients with resolved HBV infection.** PBMC and PBMC depleted of CD4+CD25+ cells from subjects who resolved HBV infection (n=3) were stimulated with HBV (core 18-27) and Melan-A 26-35 peptides. After 8 days of in vitro expansion HBV-and Melan-A specific CD8+ cells were visualised with tetramers (Tc18-27 and T Melan-A). 5a). Dot plots are representative of the results obtained in the 3 patients. Cells were gated on CD8+ live cells. 5b) Panels show the frequencies of core18-27 and Melan-A 26-35 specific CD8 cells obtained in cells expanded from total PBMC and after depletion of CD4+CD25+ cells.
Table I-Clinical and virological features of HLA-A2+ chronic hepatitis B patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age</th>
<th>HLA-A2</th>
<th>HBe Ag</th>
<th>Anti HBe</th>
<th>HBV-DNA copies/ml</th>
<th>ALT (U/L)</th>
<th>Liver histology</th>
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<tr>
<td>Pt1</td>
<td>22</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2.4 x 10^4</td>
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<td>ND</td>
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<tr>
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<td>+</td>
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</tr>
<tr>
<td>Pt7</td>
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<td>+</td>
<td>-</td>
<td>5.8 x 10^8</td>
<td>66</td>
<td>Min NI Min fibr.</td>
</tr>
</tbody>
</table>

Legends: Min= minimal, Mod= moderate, Sev= severe, fibr.= fibrosis, NI= necroinflammation
ND.= not done
1A

[Plot showing CD4+CD25+ cell percentages with a 2.1% area highlighted.]

1B

[Scatter plots showing % CD4+CD25+ cells across different categories.]

1C

[Graph showing % CD4+CD25+ cells over time (Before, During, After).]

**HBV-DNA (copies/ml)**
- > 10^7
- > 10^7
- < 10^6

**ALT (U/L)**
- < 70
- > 70
- < 70

**HBV chronic patients**
3a

PBMC

-CD4+ CD25+

No pept  + pept

No pept  + pept

0.05%  0.6%

0.03%  1.8%

Anti-CD8

Anti-IFN-gamma

3b

HBeAg- / HBV-DNA < 10^7

HBeAg+ / HBV-DNA > 10^7

HBV-Specific

Mel-a-Specific

% Ag-specific CD8+ cells

Pt 1

Pt 2

Pt 3

Pt 4

Pt 5

Pt 6

3c

% Tc18-27 CD8+ cells

PBMC  PBMC  PBMC

PBMC -CD4 CD25+  PBMC -CD4 CD25+  PBMC -CD4 CD25+  + CD4+ CD25+