Gene Expression Signatures of Peripheral CD4+ T Cells Clearly Discriminate Between Patients with Acute and Chronic Hepatitis B Infection

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CD4+ T and regulatory T cells (Tregs) seem to play a key role in persistence of hepatitis B virus (HBV) infection. However, the molecular events by which Tregs exert their modulatory activity are largely unknown. The transcriptional profiles of CD4+ T cells of healthy controls (HCs) and patients affected by acute hepatitis B (AVH-B) or chronic hepatitis B (CHB) infection were established using a custom expression array consisting of 350 genes relevant for CD4+ T cell and Treg function. These studies were complemented by real-time reverse-transcription polymerase chain reaction. Peripheral blood mononuclear cells (PBMCs) were also analyzed for the presence of Tregs, which were more abundant in the acute stage of the disease (7%) than in HCs and CHB infection (HCs versus AVH-B, $P = 0.003$; AVH-B versus CHB, $P = 0.04$). One hundred eighteen genes (34%) intrinsically differentiate HBV-infected patients from HCs. Using gene ontology, we identified T cell receptor signaling and clusterization, mitogen-activated protein kinase kinase signaling, cell adhesion, cytokines and inflammatory responses, cell cycle/cell proliferation, and apoptosis as the most prominent affected modules. A higher expression of CCR1, CCR3, CCR4, CCR5, and CCR8 was seen in AVH-B than in CHB-infected patients and HCs. Annotation of the interconnected functional network of genes provided a unique representation of global immune activation during acute infection. Almost all genes were down-regulated in patients with CHB infection. *Conclusion:* The fingerprints enable clear discrimination between patients suffering from AVH-B or CHB infection. The observed profiles suggest accumulation of effector T cells with a potential role in necro-inflammation during the acute stage. Subsequent down-regulated effector functions support the hypothesis of suppressed CD4+ effector T cells favoring viral persistence in the chronic infection stage. (HEPATOLOGY **2009;49:781-790.**)

Hepatitis B virus (HBV) infection is one of the major infectious diseases; more than 360 million people worldwide have chronic hepatitis B (CHB) infection. Acute hepatitis B (AVH-B) infection does not get cleared by the host immune system in about 5% of adult patients, leading to the development of CHB.[1]

HBV infection is a dynamic process, and the outcome of infection varies due to differences in host response.

Abbreviations: ANOVA, analysis of variance; AVH-B, acute hepatitis B infection; CHB, chronic hepatitis B; GO, gene ontology; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HC, healthy control; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MAPKK, mitogen-activated protein kinase kinase; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse-transcription polymerase chain reaction; TCR, T cell receptor; TNF, tumor necrosis factor; Treg, regulatory T cell.

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Some subjects with CHB remain asymptomatic, even after many decades of infection, with slow disease progression, whereas others rapidly progress to cirrhosis and hepatocellular carcinoma. A strong genetic component with modified gene expression seems to be a major driving force affecting the course of viral hepatitis.[2-5]

Development of persistent infection following AVH-B is associated with T cell hyporesponsiveness and dysfunction.[6] Recently, the role of CD4+CD25+ regulatory T cells (Tregs) has been shown in persistent viral infections.[7-11] The virus-dependent induction of Tregs may have two different outcomes depending on the specific balance. On the one hand, it may be an important process to limit excessive immunopathological damage during viral clearance. On the other hand, it could also facilitate viral persistence by down-modulating critical effector cells.[12,13] Depletion of CD4+CD25+ T cells in herpes simplex virus–infected mice resulted in enhanced virus-specific CD8+ T cell activity in the acute phase of infection, as well as after viral clearance. Insufficient CD8+ effector cell response was also observed in chronic infection with the Friends virus, as a result of Treg accumulation at the site of infection.[14,15]

Recent studies of HBV confirm the important role of Tregs in the outcome of viral infection. A broad analysis of the frequency and function of CD4+CD25+ Tregs in the blood with AVH-B, CHB, severe CHB, and HCs revealed that patients with severe CHB infection showed significantly higher number of Tregs compared with patients with mild CHB and early AVH-B infection, and that CD4+CD25+Foxp3+ T cells accumulate in the livers of patients with severe CHB infection. In convalescent AVH-B patients, the number of circulating Tregs was increased to normal levels after viral clearance.[8,11] These data suggest that Tregs play a pivotal role in the modulation of T cell responses against HBV during infection, thereby influencing the outcome of the disease.

Although, some progress has been made in the characterization of AVH-B and CHB at the cellular level, there is a paucity of knowledge about the underlying molecular mechanisms and specific genes affected during host response against HBV. Thus, we performed a comparative evaluation of CD4+ T cells purified from peripheral blood of patients with AVH-B and CHB infection in comparison to HCs. To gain insight on how the status of infection influences the overall expression pattern of CD4+ T cells, we analyzed their transcriptional profiles using microarray technology. We used a focused gene array consisting of 350 CD4+ T cell–specific genes, customized to investigate Treg-associated genes.[16]

Patients and Methods

Patients and Samples. Three groups of subjects were included in the present study (Table 1). The first group comprised 19 patients with AVH-B diagnosed by clinical symptoms of acute hepatitis and serum alanine aminotransferase levels >10 times the upper limit of normal with no past history and clinical, biochemical, or radiological evidence of chronic liver disease. These patients were hepatitis B surface antigen (HBsAg)-positive, hepatitis B e antigen (HBeAg)-positive, anti-HBeAg–negative, immunoglobulin (Ig) M anti-hepatitis B core antigen (HBcAg)-positive and anti-HBsAg–negative. The second group comprised 21 HBsAg-positive CHB patients for >6 months, HBeAg positive, with elevated alanine aminotransferase levels (>1.5 times the upper limit of normal) and histological evidence of chronic hepatitis. The third group comprised 12 HCs with no previous history or current evidence of any liver disease, normal aminotransferase levels, normal liver on ultrasound, and negative for HBsAg, anti-HBeAg, IgG anti-HBcAg, anti-HCV, IgM anti–hepatitis E virus, IgM anti–hepatitis A virus, and anti–human immunodeficiency virus. All HCs had received vaccination for HBV in the past, had anti-HBsAg titers >300 IU/mL, and were negative for total antiHBc.

Exclusion criteria were regular alcohol consumption (>40 g/day for the past 5 years), diabetes, severe systemic illness, pregnancy, hepatocellular carcinoma, coinfection

Table 1. Clinical and Virological Characteristics of the Subjects Enrolled in the Study

Parameters	AVH-B (n = 19)	CHB (n = 21)	HC (n = 12)	*P* Value*	
				AVH-B Versus HC	CHB Versus HC
Age (years), mean ± SD	29.42 ± 14	29.4 ± 11	29 ± 5	NS	NS
Sex (M:F)	13:6	18:3	9:3	NS	NS
Serum bilirubin (mg/mL), median (range)	10.5 (0.5–33.6)	0.7 (0.2–23.8)	0.3 (0.1–0.7)	*P*=0.026	NS
Albumin (g/dL), median (range)	3.95 (2.2–4.8)	4.1 (2.7–4.8)	4 (3.5–4.2)	NS	NS
AST (IU/L), median (range)	605 (52–2,498)	66 (36–551)	33 (30–35)	*P*= 0.02	*P*=0.015
ALT (IU/L), median (range)	1,170 (470–4,329)	121 (55–204)	30 (30–34)	*P*= 0.02	*P*=0.000

Abbreviations: F, female; M, male; NS, not significant; SD, standard deviation.

*The *P* value was calculated for both AVH-B patients versus HCs and CHB patients versus HCs.

with human immunodeficiency virus or other hepatitis viruses, or immunosuppressive therapy for other associated illness. After institutional ethical committee approval, informed consent was obtained in all cases before enrolling the patients for conducting the various blood tests.

HBsAg, HBeAg, anti-HBeAg, IgM anti-HBcAg, IgG anti-HBcAg, anti-HBsAg, IgM anti–hepatitis A virus, IgM anti–hepatitis D virus, IgM anti–hepatitis E virus, anti–human immunodeficiency virus, and anti-HCV were detected using commercial enzyme-linked immunosorbent assay kits (M/s Ranbaxy Diagnostics, India).

Twenty-milliliter blood samples were collected from patients with acute HBV at the first presentation, with a mean duration of 33.5 ± 13.1 days after the onset of jaundice. In the other groups, routine fasting blood samples were collected. After isolation of peripheral blood mononuclear cells (PBMCs), CD4+ T cells were isolated. For microarray experiments, equal numbers of CD4+ T cells (100,000 cells) from each patient were pooled and total RNA was isolated for further experiments. For quantitative reverse-transcription polymerase chain reaction (RT-PCR), individual CD4+ T cells were isolated and total RNA was purified. A minimum of 1 to 2 μg of total RNA was used to make complementary DNA for RT-PCR.

Isolation of CD4+ T Cells from PBMCs and Flow Cytometric Analysis. PBMCs were separated by Ficoll-hypaque density gradient centrifugation. CD4+ T cells were isolated using an indirect magnetic labeling method (Miltenyi Biotec, Germany) and were checked for purity after staining with anti-CD4 fluorescein isothiocyanate and PeCy7–anti-CD3 or APC–anti-CD4 and fluorescein isothiocyanate–anti-CD25 anitbodies (BD Pharmingen). Permeabilized cells were fixed using Cytofix/Cytoperm (BD Pharmingen) and subsequently stained with phycoerythrin–anti-FoxP3 (eBiosciences). Cells were acquired for flow cytometric analysis using FACSCalibur, and the results were analyzed using Flow-Jo software.

Microarray Analysis. To normalize results within each individual group, total RNA was extracted from pooled CD4+ T cells (n = 5 per group) using the Qiagen RNA extraction easy kit. The quality and integrity of the total RNA isolated from CD4+ T cells pools were controlled by running the samples on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). For microarray analysis, we used the human Treg Chip as described elsewhere.[16]

Quantitative RT-PCR was performed in triplicate in a 7900 ABI Prism Sequence Detection system using the Syber Green kit and specific primers (Table 2) for NCF4, MCM5, NFKB, CCR1, CCR3, and CCR5 with Primer Express 1.5 software (Applied Biosystems). Amplification of β-actin was used as the control for normalization. Relative quantification of each gene was analyzed by calculating the Log RQ of each sample Ct value.

Table 2. Primer Sequences Used in Real-Time RT-PCR

Gene	Primers
NCF4	5′-TGAACAGCTTCCGGATGATG-3′
	5′-TGAAGCCTCTCTTCTCCTCGAT-3′
MCM5	5′-ACCCTGGGACAGCCAAGTC-3′
	5′-CTGCCTTTCCCAGACGTGTATAC-3′
NFKB	5′-TGATCACCAACCAGCCAGAA-3′
	5′-TCTCGGAGCTCAGGATCACA-3′
CCR1	5′-ACACAGGCTTGTACAGCGAGATC-3′
	5′-AAACACGGCGTGGACGAT-3′
CCR3	5′-TGCTACACAGGAATCATCAAAACG-3′
	5′-GAGCCGGATGGCCTTGTA-3′
CCR5	5′-GAATATGAACGGTGAGCATTGTG-3′
	5′-ACAACTTAAGAGCAAAAGGAAAGACAT-3′
β-Actin	5′-CCAGCTCACCATGGATGATG-3′
	5′-ATGCCGGAGCCGTTGTC-3′

Statistical Analysis. Signal intensities were qualified and quantified using the Imagene software 5.0 (BioDiscovery, El Segundo, CA) and normalized according to the housekeeping controls. Genes whose signal intensities did not exceed background levels (signal intensity was less than background intensity plus two times standard deviation) were excluded from further analysis. Significant differentially regulated genes between AVH-B patients, CHB patients, and HCs were identified via analysis of variance (ANOVA) at a 95% confidence level. Multiple testing errors were taken into account and corrected using the Benjamini-Hochberg algorithm.[17] For data visualization in two-dimensional hierarchic clusters, the Genesis software v1.4.0 was applied.[18] Genes were annotated for gene ontology (GO) terms using the Source annotation tool[19] and linked to external signaling maps by GenMapp.[20,21] The statistical significance of intergroup differences for continuous variables was evaluated by means of Mann-Whitney U tests and for categorical variables with chi-square tests. A P value < 0.05 was considered significant.

Results

Frequency Analysis of Circulating CD4+CD25+ Foxp3+ Tregs. We analyzed peripheral blood from 19 AVH-B patients, 21 CHB patients, and 12 HCs to determine the percentage of CD4+ T cells and CD4+CD25+Foxp3+ Tregs. The clinical characteristics of the patients and the HCs enrolled in this study are shown in Table 1. The frequency of CD4+ cells was similar in all the three groups (AVH-B, 29%; CHB, 34%; HC, 30%) (Fig. 1A). The frequencies of Tregs in HCs and in chronic infection were 2.18% and 3.96%, respec-

tively, whereas in AVH-B patients it was 7% (HC versus AVH-B, $P = 0.003$; AVH-B versus CHB, $P = 0.04$) (Fig. 1B).

Functional Dissection of Expression Signatures in AVH-B and CHB Patients. CD4+ T cells were isolated from peripheral blood (92% to 96% purity) for RNA extraction. Equicellular donor pools derived from randomly selected (n = 5) AVH-B, CHB, or HC donors were created to minimize overestimation of individual expression signatures. To minimize systemic errors based on variances during RNA processing steps and hybridization, we performed four (three for AVH-B) individual measures per donor pool. A unique set of 350 genes known to be crucial for Treg and CD4+ effector T cell function was used to establish an expression profile of CD4+ cells. Significant expression levels could be detected for 316 genes. A summary of this expression data is provided in Supplementary Table 1. Principal component analysis was used to evaluate the overall variances between and within the three groups. A clear separation of the three CD4+ pools was achieved by the principal component analysis (Fig. 2). In HCs, a higher intragroup variance was observed in comparison to the other groups. Using ANOVA and the Benjamini-Hochberg FDR algorithm at a confidence level of 95% (corrected P value < 0.05), we demonstrated that 118 out of 316 genes (37%) were differentially regulated in AVH-B and CHB patients compared with HCs (see Supplementary Table 2).

Out of 118 genes, 100 genes were assigned to GO terms using the Source annotation tool (Fig. 3A). The majority of the genes were related to cellular processes (24%), biological regulation (14%), response to stimulus (13%), metabolic processes (12%), developmental processes (11%), multicellular organismal processes (8%), and immune system processes (7%). With respect to the microarray-based analysis of the viral infection process, we further dissected the GO term "response to stimulus" (46 genes) (Fig. 3B) and "immune system process" (24 genes) (Fig. 3C).

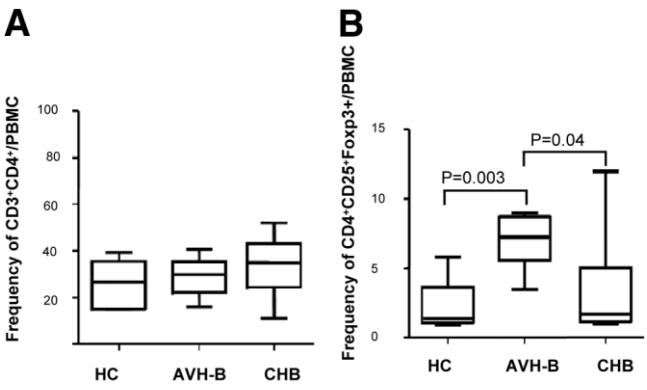

Fig. 1. Frequency of (A) CD3+CD4+ and (B) CD4+CD25+Foxp3+ T cells in peripheral blood of various subjects. Data are expressed as box plots in which the horizontal lines indicate the 25th, 50th, and 75th percentiles of the frequencies of the indicated T cells, as measured via flow cytometry. The vertical lines represent the 10th and 90th percentiles. The highest frequency of Tregs was observed in AVH-B patients (7%; HC versus AVH-B, $P = 0.003$; AVH-B versus CHB, $P = 0.04$).

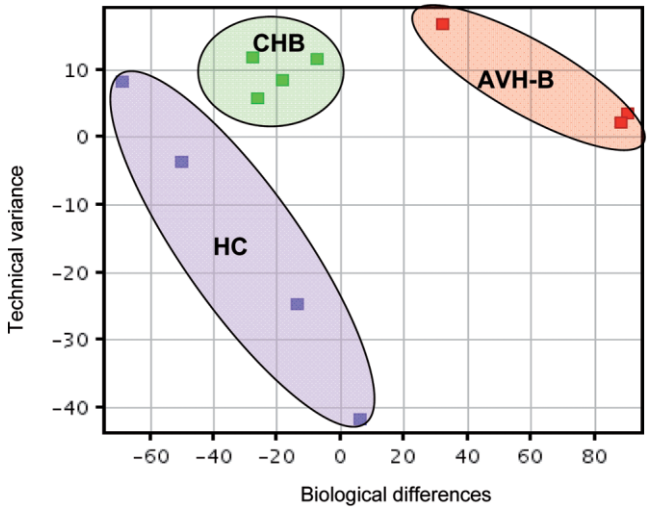

Fig. 2. Expression data obtained from the individual microarray measures were applied to principal components analysis. The use of this statistical technique allowed us to determine the key variables in our multidimensional dataset and visualize the observed variances within a group (component 1 = technical variances [y axis]) and between groups (component 2 = biological differences [x axis]), respectively. Abbreviations: HC, healthy control; CHB, chronic hepatitis B; AVH-B, acute hepatitis B.

Cluster Mapping and Affected Signaling Modules. The cluster map was organized into six sections according to the general expression behavior of the AVH-B and CHB stage of infection as compared with HCs (Fig. 4). The average relative gene expression values were calculated from four (CHB patients and HCs) and three (AVH-B patients) individual measures, respectively. Genes were strictly down-regulated in AVH-B patients (cluster 1) or in both AVH-B and CHB patients (cluster 2). Cluster 3 showed stronger expression in the AVH-B group compared with the CHB group. Cluster 4 showed significant reduction of expression of genes in the CHB group compared with HC and AVH-B patients. In cluster 5, all genes were specifically activated under acute but not chronic conditions. Cluster 6 represents genes with strong activation under both acute and chronic infection. All genes displayed in the cluster map were linked to signaling maps stored in public databases by using MAPP Finder software. T cell receptor (TCR) signaling, cell adhesion, mitogen-activated protein kinase kinase (MAPKK) signaling, cell cycle/cell proliferation, apoptosis, cytokines, and chemokines were identified as the most prominently affected modules (Fig. 5). Independently of any module,

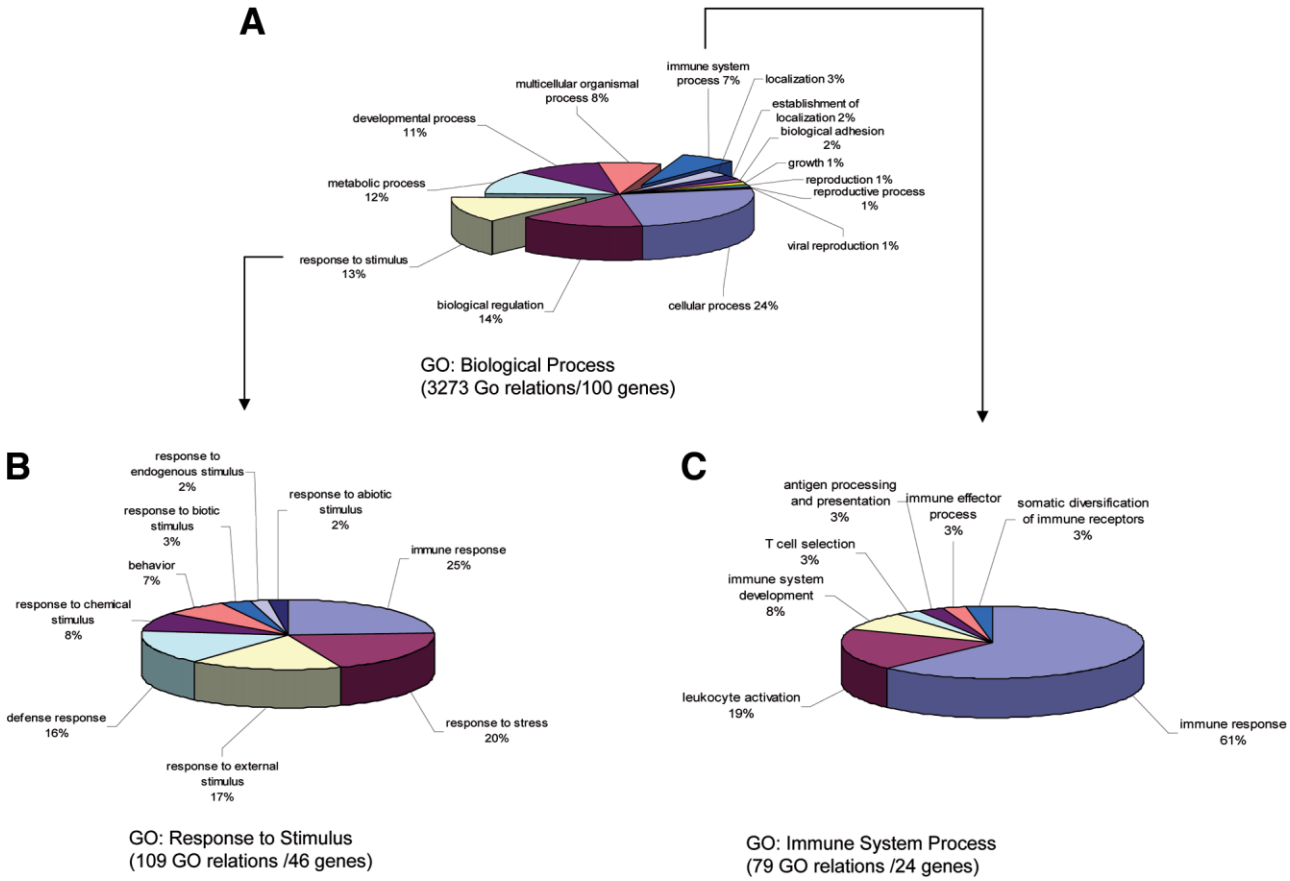

Fig. 3. Gene Ontology (GO) terms are used to identify the major functional classes of genes affected according to the signatures obtained from AVH-B, CHB, and HC expression datasets. (A) Out of 118 differentially expressed genes, 100 genes can be mapped to GO terms allocating to "Biological Process". The most frequently affected GO terms were displayed as a pie diagram. The relative frequency by which a GO term is affected through the expression signature is indicated for each section. Immunological relevant GO terms were shown as released pie sections and are further analyzed. (B) 46 genes are linked to the GO term "Response to Stimulus" and (C) 24 genes are linked to the GO term "Immune System Process". The relative impact to the most affected GO terms is indicated, as described above.

the expression of NCF4 and MCM5, which are involved in RNA and DNA replication, was up-regulated in both acute and chronic hepatitis B.

Concerning the "TCR signaling and clusterization" module, molecules such as CD4, CD3, VAV2, NCKAP, SWAP70, and CISH were activated under acute infection. Under chronic conditions, the expression of these molecules was suppressed, except for CD4 and CD3, which were equally expressed in both stages. In the context of the module cell adhesion and cell surface molecules, overexpression of ICAM1 and PECAM1 in CD4+ T cells was observed during AVH-B infection. On the other hand, ERBB2, KSR, RABAC1, RASA3, and TIAM are among the genes involved in MAPKK signaling that were up-regulated in AVH-B. All these genes were down-regulated during CHB infection. The expression of several genes involved in the regulation of the cell cycle (such as CDK4, CCND3, NEK2, PIM1, TGFB1, EGR1, and EGR2) was much higher in AVH-B samples than in those obtained from CHB patients and HCs.

During AVH-B infection, a variety of proteins involved in apoptosis were up-regulated. Overexpression of tumor necrosis factor (TNF), its ligands, and its receptors (TNFSF5, TNFSF6, TNFRSF6, TNFRSF25, TNFRSF10, and TNFSF1) were observed during AVH-B infection. Along with these genes, overexpression of the perforin genes PRF1, LTA, BAX, and ATM, which are proapoptotic, was observed during the acute stage. We also observed different expression patterns for cytokines influencing T cell activation and proliferation, as well as the balance between TH1 and TH2 cells. Interleukin (IL)-2RA, IL-7R, IL-7, IL-5, and interferon (IFN) signal-regulating cytokines (such as IL-9, IL-4, IL1R2, IFNA1, and SOCS2) were up-regulated in AVH-B infection. Microarray analysis also revealed higher expression of CCR4 and CCR8 in AVH-B patients than in CHB and HC.

Quantitative RT-PCR. Two representative up-regulated genes, NCF4 and MCM5, were validated in an independent set of 14 AVH-B and 16 CHB patients and in 12 HCs via quantitative RT-PCR. An increased expres-

sion of NCF4 and MCM5 was observed in hepatitis patients compared with HCs (Fig. 6). There was no significant difference between the expressions of NCF4 in all patient categories. However, MCM5 expression was higher in AVH-B patients than in CHB patients. These RT-PCR results were in full agreement with the microarray data (Supplementary Table 1), which also showed up-regulation of CCR5 and CCR8. However, other common chemokine receptors, such as CCR1 and CCR3, were not present on the customized chip. Therefore, we expanded our studies by analyzing the CCR1, CCR3, and CCR5 expression profiles via RT-PCR. An increased expression of CCR1, CCR3, and CCR5 was observed in samples from AVH-B and CHB patients as compared with HCs (Fig. 6). RT-PCR data also showed increased expression of NFKB in AVH-B and CHB patients. This is in functional agreement with the down-regulation of the NFKBIA gene, an inhibitor of NFKB, which was detected in the microarray studies.

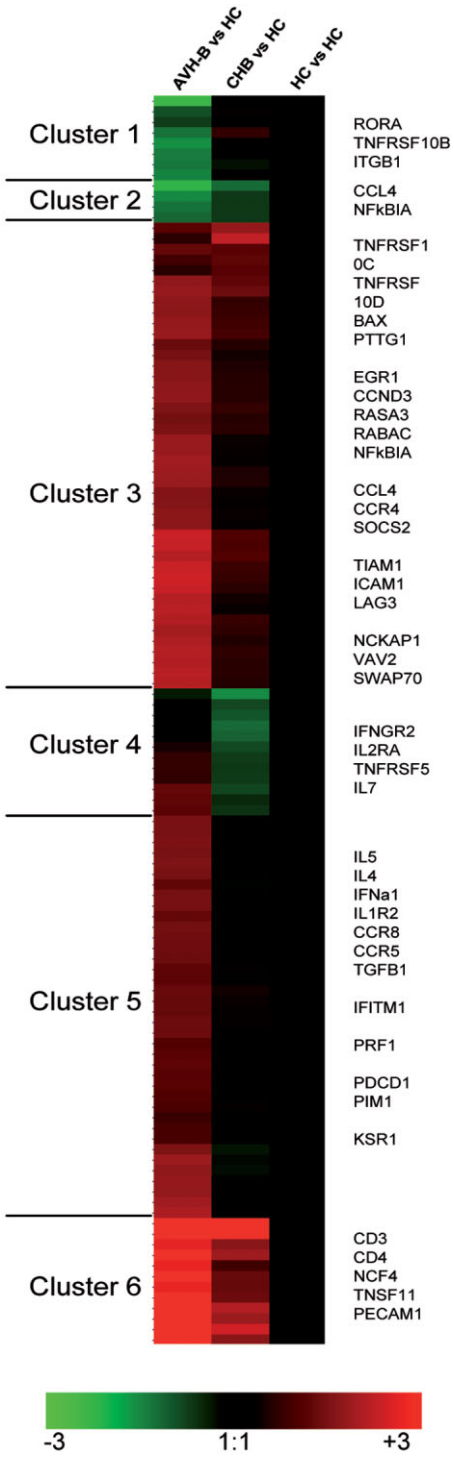

Fig. 4. Averaged expression signature of CD4+ T cell pools isolated from AVH-B and CHB patients as compared with HCs. One hundred eighteen genes are shown passing ANOVA at a 95% confidence level (internal controls were excluded) (Supplementary Table 2). Each lane represents the average signal log intensity of three (AVH-B) and four (CHB and HC) independent technical replicates. Multiple testing errors were taken into consideration and corrected using the Benjamini-Hochberg algorithm. Relative expression was displayed in red for increased expression levels, green for decreased expression levels, and black for no changed expression levels as compared with HCs. Color intensity was calibrated to expression level as illustrated in the color legend at the bottom of the cluster map. Cluster dissection is indicated on the left-hand side; representative genes for each cluster are indicated on the right-hand side.

Discussion

Differences in host immune response significantly affect the outcome of HBV infection, Tregs being a major player in the fine-tuning of T cell function. In contrast to other studies,[8,22] we found the highest number of Tregs in AVH-B patients (7%), whereas the frequency was only 4% in CHB patients. Nevertheless, this value was still two-fold higher than the percentage of Tregs in HCs (2%) (Fig. 1B). It was described that during the convalescence phase of AVH-B infection, the number of Tregs increases before the normal level is restored after resolution of the infection.[22] This dynamic modulation of the antiviral immune response via self-limited action of suppressor Tregs might support the healing process at the site of infection. The activity of Tregs might prevent the extended tissue damage resulting from an unchecked proinflammatory response. On the other hand, a spatiotemporal dysregulation of Treg activity—that is, uncontrolled establishment of suppressor activity at the site of infection—could promote viral persistence and progression to chronicity. This hypothesis is supported by the increased number of circulating Tregs measured in CHB patients as compared with HCs. The accumulation of Tregs in the liver accompanied by viral persistence illustrates the impact of Tregs on immune suppression.[22] However, the molecular events are largely unknown.

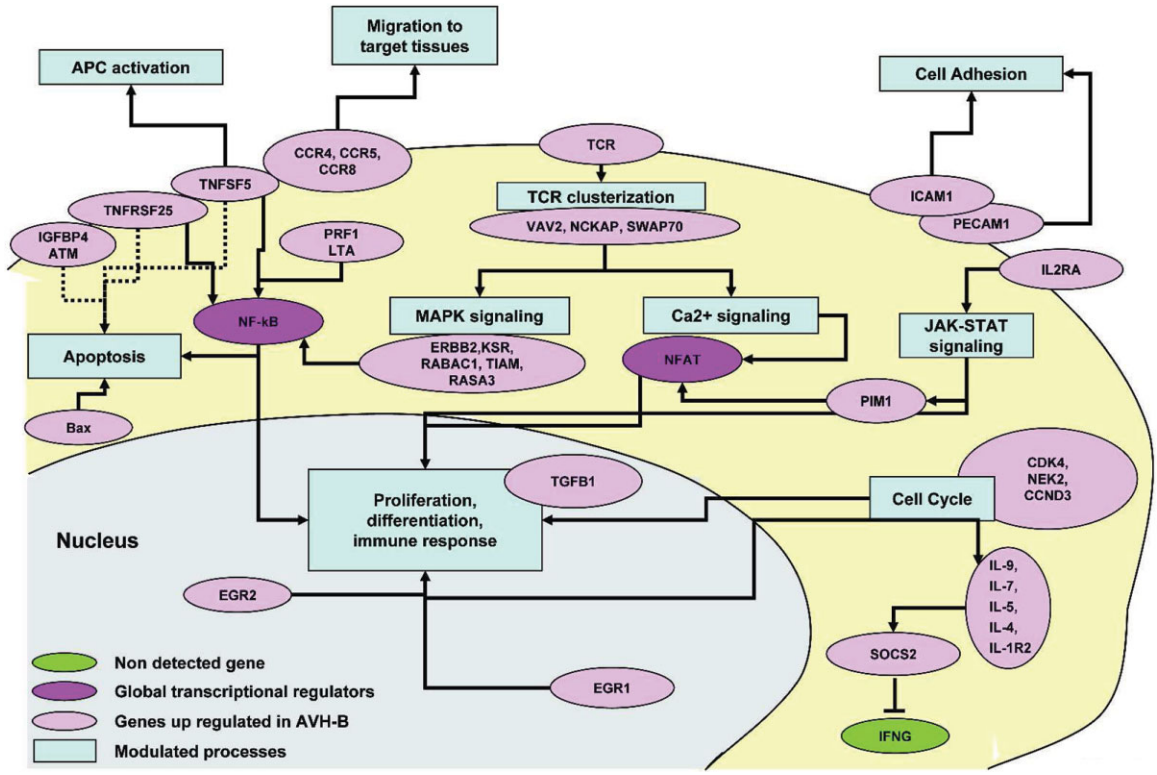

Fig. 5. Signaling module depicting gene sets activated in CD4+ T cells from AVH-B patients, which were attenuated or restored to normal levels during the chronic stage of infection. Dotted lines represent a minor effect on gene expression or the specific process.

Microarray technology has a tremendous potential for complementing classic biological and clinical parameters; therefore, we used a human Treg chip[16] to dissect specific gene networks affected by the action of Tregs on CD4+ T cells, which may contribute to the pathogenic stages of acute and chronic HBV infection. To gain better insight into this process, it would be necessary to study the gene expression profiles of AVH-B adult patients who later develop chronic infection. However, an extremely large prospective patient cohort will be needed, because less than 5% will develop chronic infection. To overcome this bottleneck, we enrolled chronic HBV-infected adults who are age- and sex-matched for the AVH-B patients. Children were excluded, because they might be more immunotolerant.

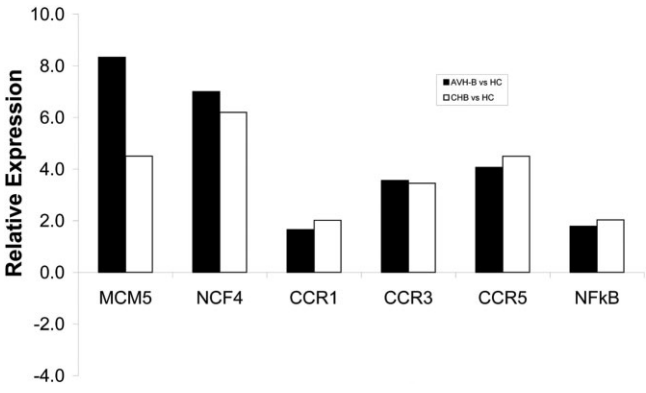

Fig. 6. Quantitative RT-PCR analysis of selected genes. MCM5, NCF4, and CCR5 were used to confirm the results of microarray experiments. The expression levels of CCR1, CCR3, and NF-κB, which are known to play an important role in infection processes, were also analyzed. Results indicate relative expression levels as ratios of normalized mean gene expression of infected patients as compared with HCs.

Expression Signatures and Affected Signaling Modules. Expression of the NCF4 and MCM5 genes, which are RNA and DNA replication genes, was up-regulated in both AVH-B and CHB infection. Although not directly attributable, NCF4 is known to favor the amplification of viral RNA,[23] whereas MCM5 is essential for the induction of STAT1 in response to IFN-α stimulation and is involved in DNA replication and transcription.[24]

Out of 118 genes, 100 genes were assigned to GO term using the source annotation tool. Combining the results from functional and expressional clusterization, we found a large set of genes involved in immune response processes activated under AVH-B infection, which were attenuated or restored to normal levels during the chronic stage of infection (Figs. 4 and 5). MAPP Finder software was used for linking expression profiles to signaling maps stored in public accessible databases[20,21] and constructed the signaling modules (Fig. 5). There is a considerable number of genes acting not only in a particular module but also

cross-linking the individual modules. This highlights the overall complexity of the underlying signaling modules.

TCR Signaling and Clusterization. Fully competent activation of T cells is mediated by the TCR, which in turn facilitates the accumulation of TCR accessory molecules, thereby forming a structure known as immunological synapse. Molecules such as VAV2, NCKAP, SWAP70, or CISH are part of this signaling complex or are directly affected by this complex. Dysregulation or mutation of these genes severely influences the outcome of the immune response.[25-29] We observed activation of these components under acute infection, suggesting an enhanced outside/inside signaling. Under chronic conditions, the expression of these molecules was suppressed, except for CD4 and CD3, thereby indicating an attenuation of the signal transfer into the cell. The signal induced upon activation of the TCR complex can be transmitted to several other signaling modules, such as activation of MAPKK cascades, NF-κB-mediated control of cell proliferation, and activation of Ca2+ release resulting in NFAT migration into the nucleus (Fig. 5).

Cell Adhesion and Cell Surface Molecules. Analysis of the expression of cell adhesion and surface molecules distinctly recognize the patterns of lymphocyte trafficking in acute and chronic inflammations. ICAM1 and PECAM1, which are overexpressed in the acute stage, have shown their essential role for membrane trafficking and transendothelial migration of activated leukocytes.[30,31]

MAPKK Signaling. ERBB2, KSR, RABAC1, RASA3, and TIAM are among the genes involved in MAPKK signaling that were up-regulated in AVH-B patients. These genes are part of the mitogen-activated protein kinase and are involved in the modulation and stimulation by growth factors. Sequestration of the proto-oncogene ERBB2 into an ERBB2 growth factor receptor triggers the receptor complex to relay signals inside the cell, thereby promoting cell growth, cellular survival, and inflammation.[32] A potential mechanism for ERBB2 activation of nuclear factor κB (NF-κB) is the phosphatidyl inositol 3-kinase pathway, as documented in mammary epithelial cells.[33] However, the molecular mechanisms by which all these molecules modulate the expression of cell cycle genes and cell cycle progression warrant further investigation.

Cell Proliferation and Regulation of Cell Cycles. The expression of several genes involved in the regulation of the cell cycle, including CDK4, CCND3, NEK2, PIM1, TGFB1, EGR1, EGR2, MCM5, and ATM, was much higher in AVH-B samples than in those obtained from CHB patients and HCs. Regulation of cell cycle and mitosis onset is mainly dependent on the activation of cyclin-dependent kinases such as CDK4 and CCND3 (Fig. 5) and serine/threonine protein kinases such as NEK2 and PIM1.[34] Other molecules controlling proliferation, differentiation, and mitogenesis are TGFB1, EGR1, and EGR2.[35-38]

Apoptosis. Once the antigen is cleared, most of the T cells die, and only a few are thought to remain as memory cells. Activation-induced cell death is one of the main mechanisms for T cell contraction in vivo. During AVH-B infection, a variety of proteins involved in apoptosis (such as TNFRSF25, TNFRSF10, TNFRSF5, TNFRSF6, and TNFSF11) were up-regulated. TNF receptors are main components of the extrinsic pathway of apoptosis, and NF-κB activation and cellular apoptosis are regulated through these receptors.[39] Overexpression of TNF and its receptors may stimulate the acute phase reaction and play a role in regulating lymphocyte homeostasis. Along with these genes, overexpression of the perforin gene PRF1 has also been documented in TNF-linked NF-κB signaling and apoptosis.[40,41] Other overexpressed genes in AVH-B includes LTA, BAX, and ATM, which are proapoptotic, and have multiple functions in cell-mediated cytolysis and apoptosis.[42-44] The IGFBP4 gene, which is known for inducing NF-κB-dependent increased expression of BAX and BCL2 in apoptosis,[45] was also overexpressed in the acute stage.

Cytokines and Chemokines. Different expression patterns for cytokines influence T cell activation, as well as the balance between TH1 and TH2 cells. IL2RA, IL-7, IL-5, and IFN signaling regulating cytokines are up-regulated in AVH-B patients. IL2RA are the receptors that bind and activate JAK proteins in the JAK-STAT pathway. Previous studies have shown high levels of IL-2 and IFN-γ in the acute phase of hepatitis.[46] In response to activation by a wide range of cytokines, SOCS proteins are activated and become involved in the inhibition of the IFN-γ-dependent inflammatory response.[47,48]

On the other hand, chemokines are the driving force for tissue-specific migration of inflammatory cells and T cell recruitment. Disproportionate expression of chemokine receptors in the affected organs provides the framework for tissue-specific recruitment of helper and cytotoxic T cell subsets. Moreover, up-regulation of CCR1-expressing CD4+ T cells to the liver was observed and treatment with a dual CCR1/CCR5 peptide antagonist, methionylated RANTES, was used to reduce hepatic injury and decrease the numbers of CD4+ T cells producing IFN-γ within the liver.[49] Up-regulation of CCR5 and other chemokines also suggests that T cells may respond to increases in virus replication by efficiently migrating into the infected liver.[50] Up-regulation of CCR5, CCR4, and CCR8 is also known for macrophage recruitment, activation, and differentiation of

Treg and TH2 effector lymphocytes.[51] We found higher expression of CCR4 and CCR8 in AVH-B patients, which also showed higher frequencies of Tregs in the periphery.

It could be argued that a study comparing patients who develop CHB infection in adulthood with those who develop AVH-B infection in adulthood would help exclude a possible overlay of patients whose infection was acquired during childhood but not detected until adulthood. However, the likelihood of the former group of patients is rather low; only about 1% of AVH-B infections acquired during adulthood are likely to become chronic, so approximately 1,000 AVH-B–infected patients would need to be followed to find 10 well-characterized CHB patients. The selection of healthy adult donors with high anti-HBs titers and negative total anti-HBc status, as done in the present study, allows a good comparison between acute HBV infections in adulthood with healthy controls.

To the best of our knowledge, differential expression of these genes has not been previously reported to be associated with the pathogenic mechanisms operating during the different stages of HBV infection. The observed up-regulation of activation markers, chemokines, inflammatory response genes, and apoptotic genes in the circulating CD4+ T cells provides the conceptual framework to support the necro-inflammation observed at the target organ as a result of effector cell accumulation, active proliferation, apoptosis, and cellular lysis in AVH-B patients. On the other hand, the clear down-regulation of these genes during the chronic stage supports the hypothesis of suppressed CD4+ effector response favoring viral persistence. The information generated in this study suggests that the in-depth investigation of the changes in major gene programs during the follow-up of AVH-B and CHB patients would allow us to elucidate the molecular pathways underlying the decision-making processes.

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