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Increased Frequency of $CD56^{Bright}$ NK-Cells, $CD3^{-}CD16^{+}CD56^{-}$ NK-Cells and Activated $CD4^{+}$T-Cells or B-Cells in Parallel With $CD4^{+}CDC25^{High}$ T-Cells Control Potentially Viremia in Blood Donors With HCV

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A detailed phenotypic analysis of major and minor circulating lymphocyte subsets is described in potential blood donors with markers of hepatitis C virus (HCV), including non-viremic and viremic groups. Although there were no changes in the hematological profile of either group, increased the levels of pre-NK cells ($CD3^{-}CD16^{+}CD56^{-}$) and a lower frequency of mature NK cells ($CD3^{-}CD16^{+}CD56^{+}$) characterized innate immunity in the non-viremic group. Both non-viremic and viremic groups displayed significantly increased levels of $CD56^{Bright}$ NK cells. Furthermore, this subset was significantly elevated in the viremic subgroup with a low viral load. In addition, an increase in the NKT2 subset was observed only in this subgroup. An enhanced frequency of activated $CD4^{+}$ T-cells ($CD4^{+}HLA\text{-}DR^{+}$) was a characteristic feature of the non-viremic group, whereas elevated $CD19^{+}$ B-cells and $CD19^{+}CD86^{+}$ cell populations were the major phenotypic features of the viremic group, particularly in individuals with a low viral load. Although $CD4^{+}CD25^{High}$ T-cells were significantly elevated in both the viremic and non-viremic groups, it was particularly evident in the viremic low viral load subgroup. A parallel increase in $CD4^{+}CD25^{High}$ T-cells, pre-NK, and activated $CD4^{+}$ T-cells was observed in the non-viremic group, whereas a parallel increase in $CD4^{+}CD25^{High}$ T-cells and $CD19^{+}$ B-cells was characteristic of the low viral load subgroup. These findings suggest that $CD56^{Bright}$ NK cells, together with pre-NK cells and activated $CD4^{+}$ T-cells in combination with $CD4^{+}CD25^{High}$ T-cells, might play an important role in controlling viremia. Elevated $CD56^{Bright}$ NK cells, B-cell responses and a T-regulated immunological profile appeared to be associated with a low viral load. ***J. Med. Virol. 81:49–59, 2009.***

KEY WORDS: HCV; blood donors; NK and NKT cells; activated T and B-cells; $CD4^{+}CD25^{High}$

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, affecting about 130 million people worldwide, corresponding to a global population prevalence of 2.2% [Global Burden of Hepatitis C Working Group, 2004].

It is accepted widely that cellular immune responses play an important role in the immunopathogenesis of HCV infection since viral clearance is associated with vigorous and multispecific HCV-specific T-cell responses during acute infection [Lechner et al., 2000b;

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Gruener et al., 2001; Thimme et al., 2001]. In contrast, in patients infected chronically, HCV-specific T-cell responses are generally weak, focused narrowly, and often dysfunctional [Chang et al., 2001; Wedemeyer et al., 2002; Ulsenheimer et al., 2003]. While the mechanisms responsible for the dysfunction in the anti-HCV innate immune response associated with viral clearance during acute infection remain unclear, recent studies suggest a major contribution of adaptive immune responses to the pathogenesis of chronic HCV infection [Sugimoto et al., 2003; Aandahl et al., 2004; Accapezzato et al., 2004; Dittmer et al., 2004].

A minority of patients infected with HCV are able to clear the virus spontaneously during acute infection, while most patients progress to chronic infection [Poynard et al., 2003]. Control of viral replication has been linked to the cellular component of the host immune response. What distinguishes a successful cellular immune response is still not fully understood. It has been demonstrated that patients who eliminate the virus spontaneously show a strong TH1-type immune response, mediated by IFN-γ and IL-2 [Tsai et al., 1997; Cramp et al., 2000], and that HCV clearance during acute infection is followed by a vigorous and multispecific $CD4^+$ and $CD8^+$ T-cell response [Lechner et al., 2000a,b; Thimme et al., 2001].

An integrated interpretation of numerous experimental findings may allow a better understanding of the immune mechanisms involved in the inability of the immune system to control chronic HCV infection. Many hypotheses have been proposed, including the inability of the host to carry out an efficient humoral and cellular response, allowing immune escape of these highly changeable viruses (quasispecies) [Guglietta et al., 2005]; the high rate of HCV replication, which would lead to the exhaustion of $CD4^+$ T-cells through the production of large amounts of HCV antigens [Kantzanou et al., 2003]; the suppression of HCV-specific $CD4^+$ T-cell expansion [Klugewitz et al., 2002]; the intrahepatic compartmentalization and induction of apoptosis of virus-specific $CD8^+$ T-cells [Crispe et al., 2000]; the production of immunomodulatory proteins by HCV [Large et al., 1999]; and the inability of the innate immune response to promote appropriate T-cell stimulation [Bertoletti and Ferrari, 2003].

The aim of this study was to carry out a detailed ex vivo phenotypic analysis of the major and minor circulating lymphocyte subsets in HCV seropositive blood donors, including those with undetectable levels of HCV-RNA, which could clarify the immunological network triggered during HCV infection and its role in determining the outcome of infection.

MATERIALS AND METHODS

Study Population

Thirty-one blood donors screened at the Hematology and Hemotherapy Foundation (HEMOBA, Salvador, Bahia, Brazil) who tested positive for anti-HCV were invited to participate in the study. The protocol was approved by the Institutional Ethics Committee of Fiocruz-Bahia. Written informed consent was obtained from all subjects who agreed to participate in the study. According to their virological status, HCV-positive subjects were separated into two subgroups: non-viremic (anti-HCV positive and undetectable HCV-RNA, $n = 12$) and viremic (anti-HCV positive and detectable HCV-RNA, $n = 19$). The age ranges in the non-viremic and viremic groups were 21–51 and 19–55 years, respectively. Males represented 7/12 (58.3%) and 14/19 (73.7%) subjects in the non-viremic and viremic groups, respectively. Individuals from the viremic group were sub-classified using a median viral load of 369,000 IU/ml as the cut-off to determine the low ($n = 9$) and high ($n = 8$) viral load subgroups.

Healthy blood donors (anti-HCV negative and undetectable HCV-RNA, $n = 18$) were included as a control group. The age range in the control group was 19–56 years, and 15/18 (83.3%) subjects were male (Table I). Among the risk factors observed within the HCV seropositive blood donors, the use of intravenous drugs and intranasal cocaine inhalation was seen particularly in the viremic group (Table I). All volunteers with a positive diagnosis were evaluated clinically at the Edgard Santos Hospital (HUPES, Salvador, Bahia, Brazil).

Blood Samples

Blood samples were collected in two 5 ml Vacutainer tubes containing EDTA (Becton Dickinson, San Jose, CA) for flow cytometric immunophenotyping and hematological analysis. An additional tube of 10 ml without anticoagulant was collected for serological and molecular testing used to diagnose HCV infection.

Laboratory Test for Diagnosis of HCV Infection

Serological methods. Tests for anti-HCV antibody were carried out by an automated third generation ELISA (Microparticle Enzyme immunoassay, MEIA System, AxSYM; Abbott Diagnostics, Wiesbanden, Germany), according to the manufacturer's instructions. A supplemental recombinant immunoblot assay (RIBA 3.0; Chiron, Emeryville, CA) was performed as a confirmation test.

Molecular analysis. A test for HCV genomic RNA (HCV-RNA) was performed using RT-PCR. Briefly, 200 µl of serum was used for RNA extraction using the Trizol LS® reagent (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. HCV-RNA was immediately transcribed into cDNA, and nested PCR was carried out using specific primers from the 5′ NC region [Koziel et al., 1992]. Positivity was detected by electrophoresis on a 1.5% routine agarose gel, and visualization of 251 bp specific PCR products was done using ethidium bromide staining under ultraviolet light. The sensitivity of this PCR was approximately 100–200 IU/ml [Krajden, 2000]. All samples with undetectable HCV-RNA were submitted

TABLE I. Study Population Characteristics

Variable	Groups		
	Healthy (n = 18), N (%)	Non-viremic (n = 12), N (%)	Viremic (n = 19), N (%)
Age	(19–56)	(21–55)	(19–55)
Gender			
Male	15 (83.3)	7 (83)	14 (73.7)
Female	3 (16.7)	5 (41.7)	5 (26.3)
HCV-RNA			
Detectable	0 (0)	0 (0)	19 (100)
Viral load[a]			
High	NA	NA	8 (47)
Low ($<3.69 \times 10^5$ IU/ml)	NA	NA	9 (53)
Genotypes			
1	NA	NA	16 (84.2)
2	NA	NA	1 (5.3)
3	NA	NA	2 (10.5)
Risk factors (RF)			
Blood transfusion	1 (5.6)	1 (8.3)	4 (21)
Intravenous drug abuser	0 (0)	0 (0)	3 (15.8)
Intranasal cocaine	0 (0)	0 (0)	4 (21)
Tattoos	0 (0)	1 (8.3)	3 (15.8)
Previous surgery	7 (38.9)	10 (83.3)	11 (57.9)
Use of glass syringe	4 (22.2)	4 (33.3)	7 (36.8)
Acupuncture	0 (0)	1 (8.3)	0 (0)
Piercing	0 (0)	1 (8.3)	0 (0)
Dialysis	0 (0)	0 (0)	0 (0)
Not declared	0 (0)	2 (16.7)	0 (0)

NA, not applicable.
[a]Median (range).

to Amplicor RT-PCR (Roche, Nutley, NJ), which has a sensitivity of 50 IU/ml. Samples with detectable HCV-RNA were further genotyped using "in-house" RT-nested PCR and RFLP analysis [Davidson et al., 1995], and viral load was determined by RT-PCR (Amplicor HCV Monitor, Roche).

SPECIFIC MONOCLONAL ANTIBODIES USED FOR IMMUNOPHENOTYPING

Mouse anti-human monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), cychrome (CY), or PE-Cy5 were used in double or triple flow cytometric platforms for immunophenotypic analysis. FITC-conjugated mAbs included anti-CD3 (HIT3a), anti-HLA-DR, DP, DQ (TU39), anti-CD40 (5C3), and anti-CD86 (FUN-1). PE-conjugated mAbs included anti-CD56 (B159), anti-CD19-(HIB19), and anti-CD25 (IL-2Rα) (M-A251). PE-Cy5 conjugated mAbs included anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), and anti-CD16 (3G8). Isotype-matched negative controls included mouse IgG_1-FITC, PE or Cy (MOPC-21), and mouse IgG_{2a}-PE (G155-178) mAbs. All mAbs were purchased from Becton-Dickinson (Mountain View, CA).

FLOW CYTOMETRIC ANALYSIS OF PERIPHERAL BLOOD

White blood cell phenotyping was undertaken according to the manufacturer's instructions with the following modifications: in 12 mm $\times$ 75 mm tubes, 50 µl samples of peripheral blood were added to 50 µl of mixture containing FACS buffer [HBSS, 10% FCS, 0.01% sodium azide (Sigma–Aldrich, St. Louis, MO), pH 7.2, 5 µl of each mAb] for mAbs specific to several cell-surface markers; the tubes were then incubated in the dark for 30 min at room temperature. Following the incubation, erythrocytes were lysed using 2 ml FACS lysing solution (Becton Dickinson Biosciences Pharmigen, San Diego, CA). The cells were washed twice with 2 ml of FACS buffer and resuspended in 200 µl of solution. The cells were immediately acquired with 30,000 events on the FACSort flow cytometer (BD Biosciences), and the data were analyzed using Cell-Quest Software (BD Biosciences). The results are expressed as a percentage of positive cells within the selected gates. Distinct gate strategies were used to analyze major and minor lymphocyte subsets as follows: lymphocytes were first selected based on their morphometric features on size and granularity dot plots. Gated lymphocytes were examined further for their immunophenotypic features on dual fluorescence dot plot distributions (Fig. 1). Analysis of NK-cell subsets was performed within $CD3^-CD16^{-/+}CD56^{-/+}$ as described previously [Vitelli-Avelar et al., 2005]. Analysis of $CD56^{Dim}$ and $CD56^{Bright}$ cells was performed within $CD3^-CD16^+CD56^+$ NK-cell subsets. NKT-cells were analyzed within $CD3^+$ gated lymphocytes. T-cell subsets, T-cell activation status, B-cells, and co-stimulatory molecules on B-cells were analyzed within gated lymphocytes. $CD4^+CD25^{High}$ T-cells were analyzed within gated $CD4^+$ T-cells.

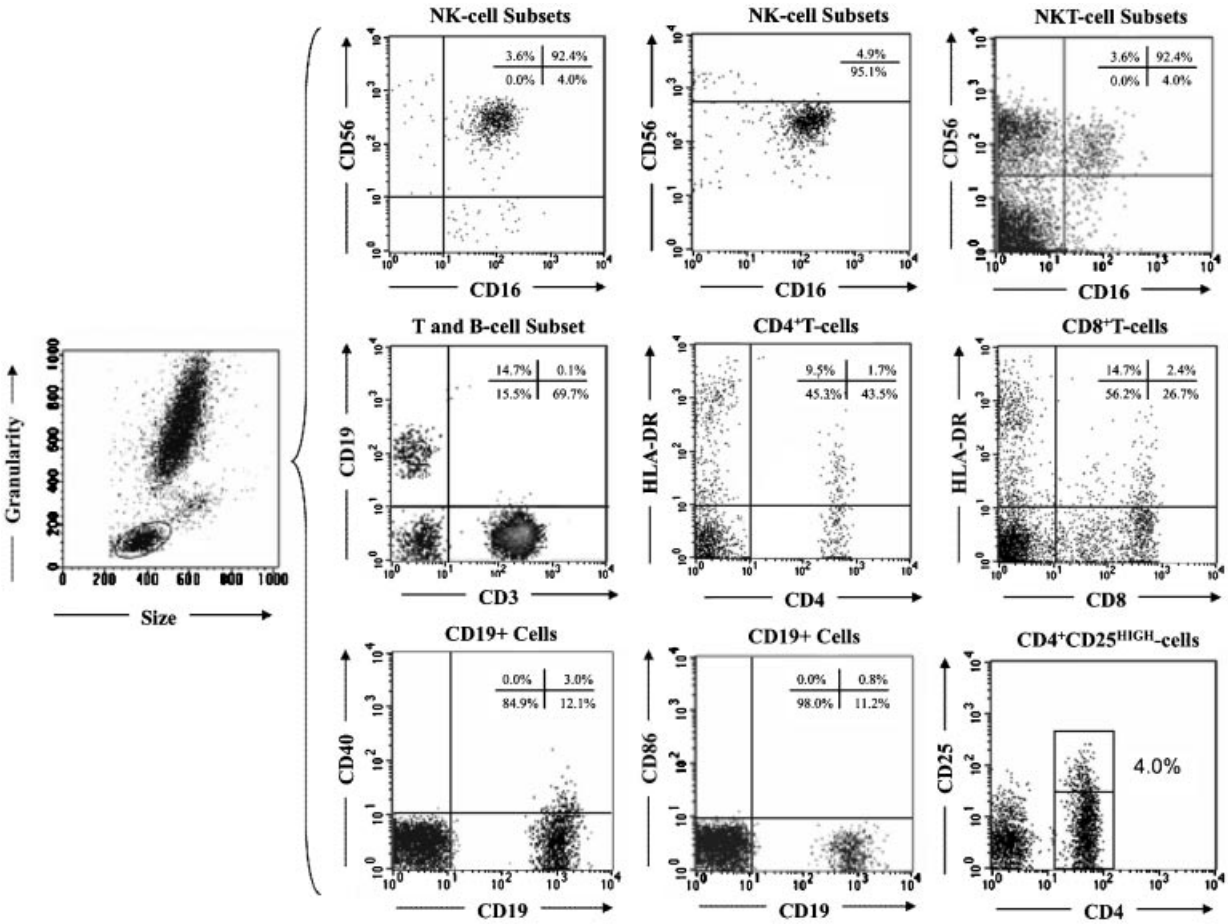

Fig. 1. Analysis of lymphocyte subsets in the peripheral blood of HCV seropositive blood donors. This panel shows representative dot plots, illustrating >40 blood donors tested.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 5.1 (GraphPad Software Incorporation, San Diego, CA). Differences among groups were first evaluated by a non-parametric Kruskal–Wallis test, followed by the Dunn's post-test to compare all pairs of data. Correlation analysis was carried out using Spearman's test. Differences were considered significant at $P < 0.05$.

RESULTS

Non-Viremic and Viremic Blood Donors Did Not Display Any Significant Changes in Their Hematological Profiles

To determine whether non-viremic and viremic blood donors showed significant alterations in their major hematological features, whole EDTA blood samples were collected, and a complete hemogram was performed using conventional automated techniques in an STKS hematology flow cytometer. Data analysis did not demonstrate any significant differences in the hematological features, including white blood cells and major leukocyte subpopulations (lymphocytes, neutrophils, monocytes, and eosinophils), as well as red blood cells, hemoglobin, hematocrit, and platelets (Table II).

Apart From a Lower Frequency of Mature NK Cells ($CD3^-CD16^+CD56^+$), Increased Levels of Pre-NK Cells ($CD3^-CD16^+CD56^-$) Were the Hallmark of Innate Immunity in Non-Viremic Blood Donors

Three major NK cell subsets have been characterized in human peripheral blood, including pre ($CD3^-CD16^+CD56^-$), mature ($CD3^-CD16^+CD56^+$), and activated ($CD3^-CD16^-CD56^+$) NK cells [Vitelli-Avelar et al., 2005]. The profile of these major NK cell subsets was characterized in the peripheral blood in non-viremic and viremic blood donors. The percentage of pre-NK cells ($CD3^-CD16^+CD56^-$) was significantly higher in the non-viremic group in comparison with the controls (Fig. 2A). Meanwhile, significantly decreased levels of mature NK cells ($CD3^-CD16^+CD56^+$) were seen in the non-viremic group compared to the controls (Fig. 2B). No differences were observed in the percentage of activated NK cells ($CD3^-CD16^-CD56^+$) among the groups studied (Fig. 2C).

TABLE II. Hematological Parameters in Blood Donors*

Parameters	Groups		
	Healthy (n = 18)	Non-viremic (n = 12)	Viremic (n = 19)
Leukocytes ($\times 10^3$/mm^3)	6.0 ± 1.2	5.5 ± 1.6	6.7 ± 2.2
Lymphocytes	2.0 ± 0.7	1.8 ± 0.6	2.2 ± 0.7
Neutrophils	3.0 ± 1.0	3.0 ± 1.1	3.2 ± 1.8
Monocytes	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 02
Eosinophils	0.4 ± 0.4	0.2 ± 0.2	0.4 ± 0.4
Red blood cells ($\times 10^6$/mm^3)	5.0 ± 0.4	4.8 ± 0.6	4.9 ± 0.4
Hemoglobin (g/L)	14.5 ± 1.0	13.8 ± 2.0	14.0 ± 1.1
Hematocrit (%)	43.5 ± 2.7	42.6 ± 3.6	42.4 ± 2.3
Platelets (counts/mm^3)	281.2 ± 5.6	256.5 ± 41.7	249.7 ± 75.7

*Results are expressed as mean values ± standard deviation.

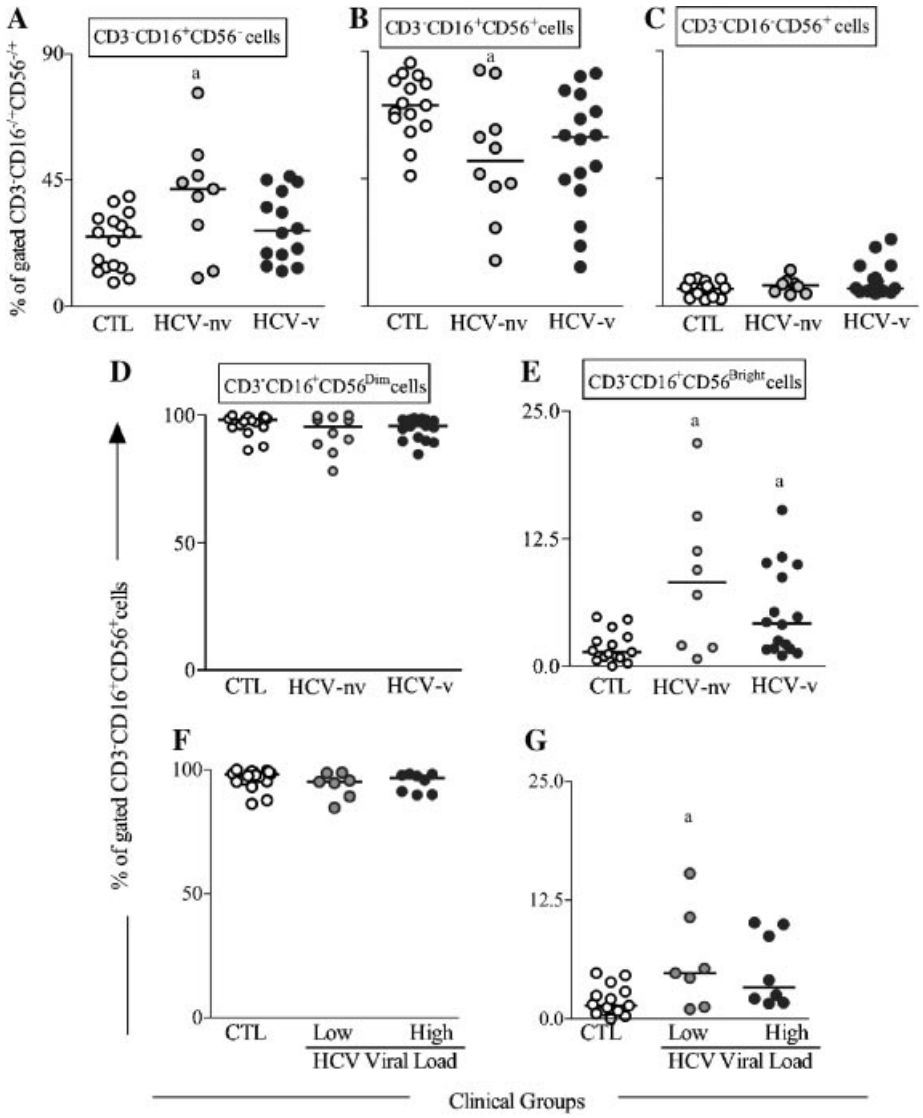

Fig. 2. Analysis of NK-cell subsets in the peripheral blood donors from non-viremic (HCV-nv ○ = 12), viremic (HCV-v ● = 19) and healthy control (CTL ○ = 18). Phenotypic studies were performed using a triple labeling flow cytometric platform to identify pre-CD3$^-$CD16$^+$CD56$^-$ (**A**), mature-CD3$^-$CD16$^+$CD56$^+$ (**B**), and activated-CD3$^-$CD16$^-$CD56$^+$ (**C**) NK cells within CD3$^-$CD16$^{-/+}$CD56$^{-/+}$ NK cells, as well as CD3$^-$CD16$^+$CD56^{Dim} (**D**) and CD3$^-$CD16$^+$ CD56^{Bright} (**E**) within mature NK cells. The individual viremic blood donors were divided based on their HCV viral load (**F**, **G**) into low (◎ = 9) and high (● = 8) viral load subgroups. Each symbol represents an individual response, and the median is shown. Significant differences at $P < 0.05$ are identified by the letter "a" in comparison with controls.

Although Both Non-Viremic and Viremic Blood Donors Displayed a High Percentage of CD56^{Bright} NK Cells, This Subset Was Increased in Those With a Low Viral Load

Cooper et al. [2001] proposed that human NK cells may be categorized into two distinct subsets according to their surface expression level of CD56. Detailed functional studies have indicated that CD56^{Dim} NK cells are more cytotoxic, while CD56^{Bright} cells produce significantly greater levels of cytokines. Aiming to focus the investigation further, a detailed analysis of these two major NK cell subsets was performed in non-viremic and viremic blood donors. No significant differences in the percentage of CD3$^-$CD16$^+$CD56^{Dim} NK cells were found among the groups studied (Fig. 2D). The findings did highlight, however, that, despite their HCV-RNA status, all HCV seropositive individuals (non-viremic and viremic) showed an increased frequency of CD3$^-$CD16$^+$56^{Bright} NK cells compared to the controls (Fig. 2E). Additional analysis taking into account the HCV viral load showed that increased levels of CD3$^-$CD16$^+$CD56^{Bright} NK cells were particularly observed in subjects with low viral load compared to controls (Fig. 2G).

Increased Frequency of Circulating NKT2 Cells of the Innate Immune System Was Observed in Blood Donors With a Low HCV Viral Load

Analysis of NKT cells was performed considering three major cell phenotypes including NKT1 (CD3$^+$CD16$^+$CD56$^-$), NKT2 (CD3$^+$CD16$^-$CD56$^+$), and NKT3 cells (CD3$^+$CD16$^+$CD56$^+$) among circulating CD3$^+$ T-cells. Data analysis showed that, despite no significant differences observed in the non-viremic and viremic groups compared to the controls (Fig. 3A–C), in viremic blood donor with a lower viral load there was an increase in the frequency of NKT2 (CD3$^+$CD16$^-$CD56$^+$) cells when compared to those with a high viral load (Fig. 3E).

Enhanced Frequency of Activated CD4$^+$T-Cells (CD4$^+$HLA-DR$^+$) Was a Distinctive Feature of Non-Viremic Blood Donors

The frequency of the major T-cell subsets (CD4$^+$ and CD8$^+$) and their activation status were evaluated within the adaptive immune compartments of blood donors with defined HCV-RNA status determined. No significant differences in the median percentage of T-cells and

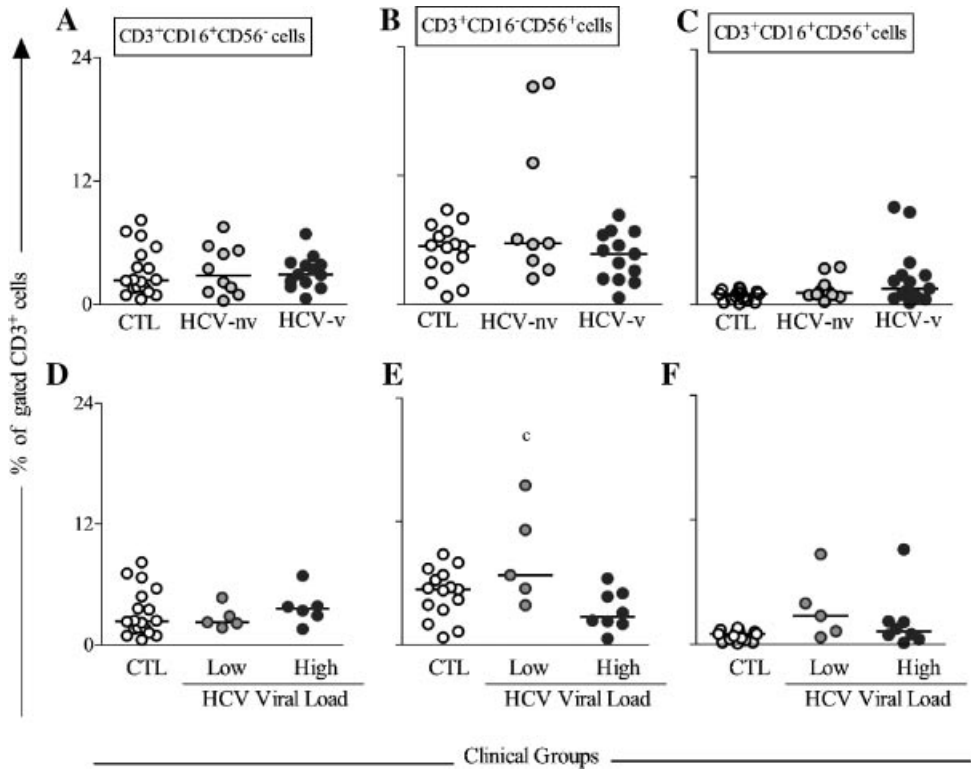

Fig. 3. Analysis of NKT-cell subsets in the peripheral blood donors from non-viremic (HCV-nv ○ = 12), viremic (HCV-v ● = 19), and healthy control (CTL ○ = 18). Phenotypic studies were carried out using a triple labeling flow cytometric platform to identify NKT1-CD3$^+$CD16$^+$CD56$^-$ (**A**), NKT2-CD3$^+$CD16$^-$CD56$^+$ (**B**), and NKT3-CD3$^+$CD16$^+$CD56$^+$ (**C**) cells within circulating CD3$^+$ T-cells. The viremic blood donors are shown based on the HCV viral load (**D**–**F**): low (◎ = 9) and high (● = 8). Each symbol represents an individual response, and the median is shown. Significant differences at $P < 0.05$ are identified by the letter "c" in comparison with the high HCV viral load subgroup.

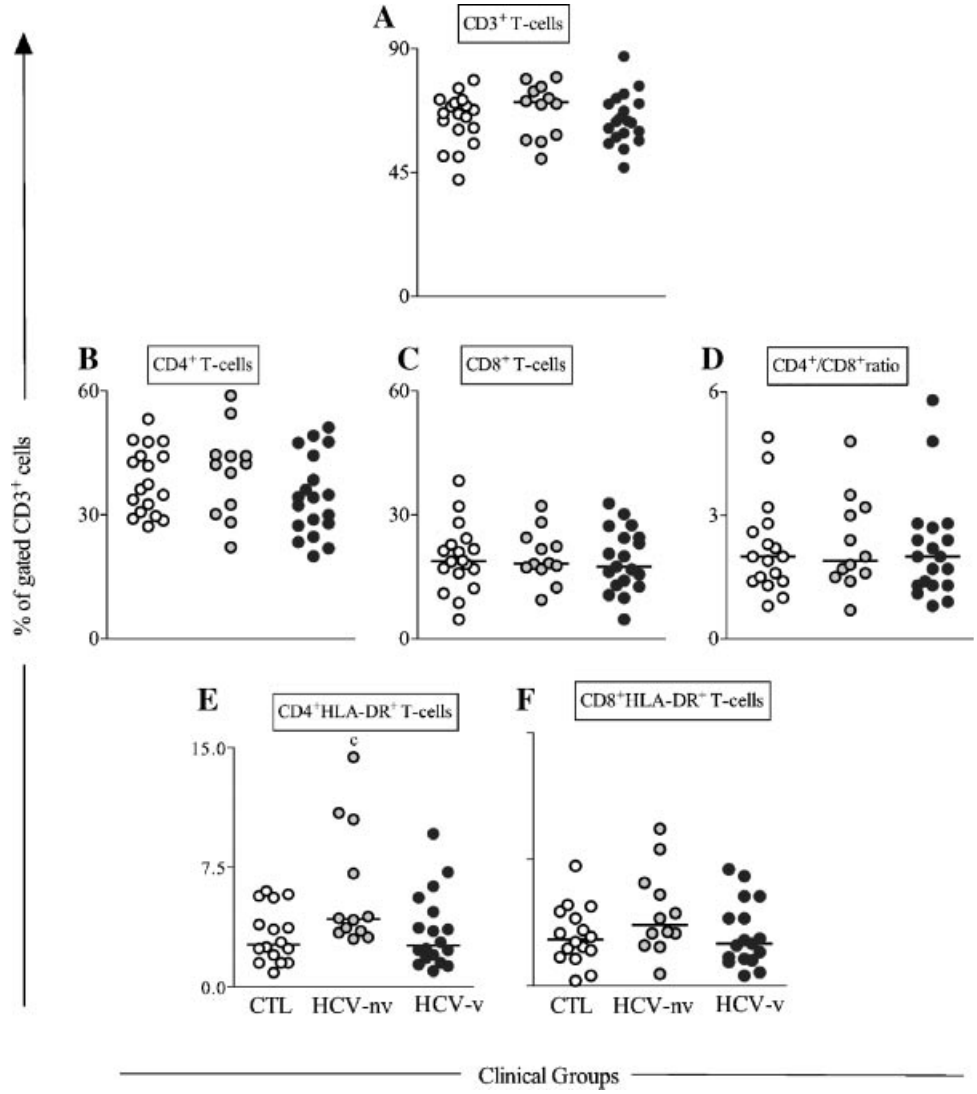

Fig. 4. Analysis of T-cell subsets and activation status in the peripheral blood donors from non-viremic (HCV-nv ○ = 12), viremic (HCV-v ● = 19), and healthy control (CTL ○ = 18). Phenotypic studies were carried out using a double labeling flow cytometric platform to identify $CD3^+$ T-cells (**A**), $CD3^+CD4^+$ T-cells (**B**), $CD3^+CD8^+$ T-cells (**C**), the $CD4^+/CD8^+$ T-cell ratio (**D**), $CD4^+$HLA-DR^+ T-cells (**E**) and $CD8^+$HLA-DR^+ T-cells (**F**) within gated lymphocytes. Each symbol represents an individual response, and the median is shown. Significant differences at $P < 0.05$ are identified by the letter "c" in comparison with the viremic group.

their major subsets were observed in non-viremic and viremic subjects compared to controls (Fig. 4A–D). Analysis of the T-cell subset activation status, however, revealed that increased levels of $CD4^+$HLA-DR^+ cells could be observed in the non-viremic group as compared to viremic group (Fig. 4E). No significant differences in the activation status of $CD8^+$ T-cells were observed (Fig. 4F). Moreover, additional analysis of T-cell subsets with regard to HCV viral load did not reveal any significant differences (data not shown).

An Increased Percentage of Circulating $CD19^+$ B-Cells and $CD19^+CD86^+$ Cells Was the Major Phenotypic Feature of Viremic Blood Donors, Particularly Those With a Low Viral Load

The percentage of $CD19^+$ B-cells was higher in the viremic group compared to controls (Fig. 5A); however, no significant differences in the median percentage of $CD19^+CD40^+$ cells were observed among the groups studied (Fig. 5C). The percentage of the $CD19^+CD86^+$ cells was higher in viremic blood donors compared to controls (Fig. 5D). Analysis of the viremic blood donors categorized according to viral load showed that increased frequency of both $CD19^+$ B-cells and $CD19^+CD86^+$ cells was a particular feature of blood donors with low viral load compared to the control group (Fig. 5B,F).

Although $CD4^+CD25^{High}$ T-Cells Were Expanded in Both the Non-Viremic and Viremic Groups, This Subset Was Predominantly Enhanced in the Viremic Subgroup With a Low Viral Load

While in murine experimental models the entire $CD4^+CD25^+$ T-cell subpopulation exhibits a regulatory

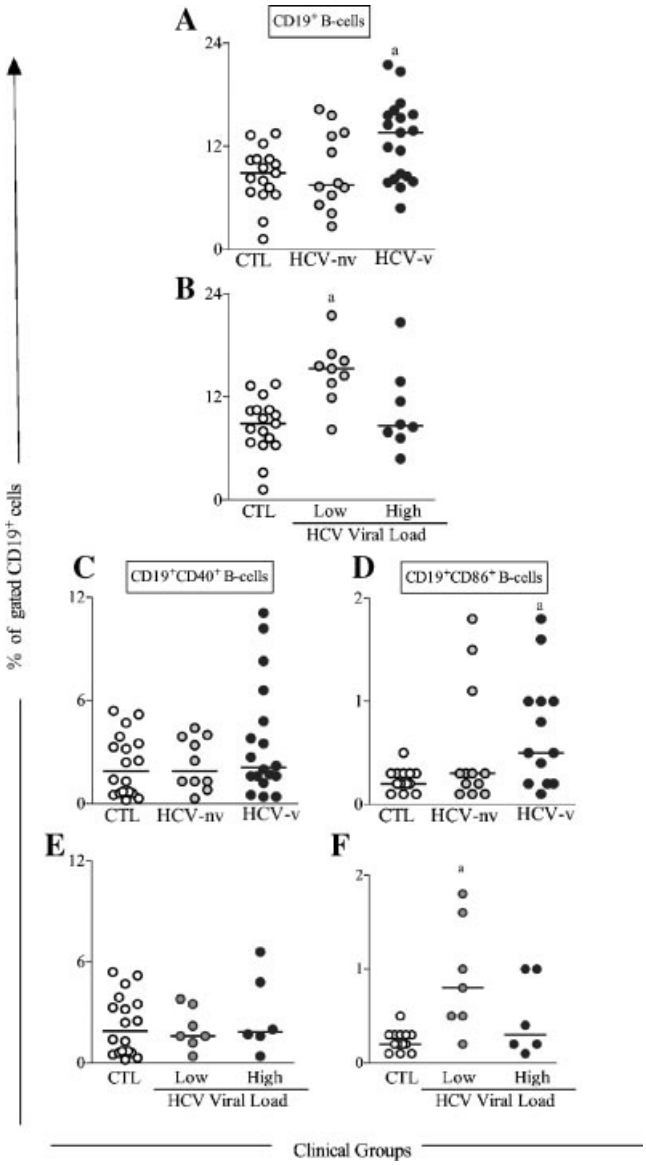

Fig. 5. Analysis of B-cells and co-stimulatory molecules on B-cells in the peripheral blood donors from non-viremic (HCV-nv ○ = 12), viremic (HCV-v ● = 19), and healthy control (CTL ○ = 18). Phenotypic studies were performed using a double labeling flow cytometric platform to identify $CD19^+$ B-cells (**A**) as well as $CD19^+CD40^+$ (**C**), and $CD19^+CD86^+$ cells (**D**) within gated lymphocytes. The viremic blood donors are shown based on the HCV viral load (**B**,**E**,**F**): low (○ = 9) and high (● = 8). Each symbol represents an individual response, and the median is shown. Significant differences at $P < 0.05$ are identified by the letter "a" in comparison with controls.

function, only the $CD4^+CD25^{High}$ cell subsets display a similar strong regulatory function in humans. The frequency of $CD4^+CD25^{High}$ T-cells was quantified in the peripheral blood of non-viremic and viremic groups. The findings demonstrated that both groups showed increased levels of $CD4^+CD25^{High}$ T-cells in peripheral blood compared to controls (Fig. 5A). Additional examination showed that the increased levels of $CD4^+CD25^{High}$ T-cells in the viremic group were observed primarily in those samples with a low viral load compared to controls (Fig. 6C).

A Parallel Increase in $CD4^+CD25^{High}$ T-Cells, Pre-NK, and Activated $CD4^+$ T-Cells Was Observed in Non-Viremic Blood Donors, While a Parallel Increase in $CD4^+CD25^{High}$ and B-Cells Was Found in the Viremic Low Viral Load Subgroup

A positive correlation between $CD4^+CD25^{High}$ T-cells, pre-NK ($CD3^-CD16^+CD56^-$) cells, and activated $CD4^+HLADR^+$ T-cells was observed in the non-viremic group (Fig. 6B). A positive correlation between $CD4^+CD25^{High}$ T-cells and $CD19^+$ B-cells was observed in the viremic group (Fig. 6B). Further analysis revealed that the frequency of $CD4^+CD25^{High}$ T-cells was correlated positively with the percentage of $CD19^+$ cells only in the low HCV viral load subgroup (Fig. 6D).

DISCUSSION

Since the discovery of HCV in 1989, attempts have been made to characterize the human immune response against the virus. Studies aimed at: (1) how patients are cured spontaneously, (2) how the virus escapes from the host immune response when infection persists, (3) what is the role of immune responses in determining the progression of the disease, (4) how effector cells are involved in the treatment response, and (5) which aspects of the immune response are critical for the development of future vaccines. The immune response to HCV infection has been studied mainly in chimpanzees, and the precise mechanism underlying the immunological events in humans is poorly understood [Su et al., 2002; Thomson et al., 2003].

The present study involved a cross-sectional investigation of major and minor changes in peripheral blood lymphocyte subpopulations during HCV infection in HCV seropositive blood donors. Viremic individuals are typically infected chronically, but they may also be infected acutely. In this situation, it is difficult to distinguish acute hepatitis C from an acute exacerbation of chronic hepatitis C [Chevaliez and Pawlotsky, 2007]. Non-viremic individuals have typically recovered from a past HCV infection. Additionally, HCV may be temporarily undetectable due to transient, partial control of viral replication before the infection becomes chronic [Lavillette et al., 2005]. This pattern cannot be differentiated from a false positive result by enzyme immunoassay (EIA) [Chevaliez and Pawlotsky, 2007].

NK cells are an important antiviral effector population, eliminating the virus by direct killing and cytokine production. Recent studies highlighting the cross-talk between NK cells, dendritic cells (DCs), and T-cells have prompted a re-evaluation of the important role that NK cells play in regulating and maintaining specific immune responses [Golden-Mason and Rosen, 2006; Kanto and Hayashi, 2007]. No changes in the hematological profiles of either group were found. A detailed phenotypic analysis of major and minor circulating lymphocyte subsets, however, demonstrated that increased levels of pre-NK cells ($CD3^-CD16^+CD56^-$),

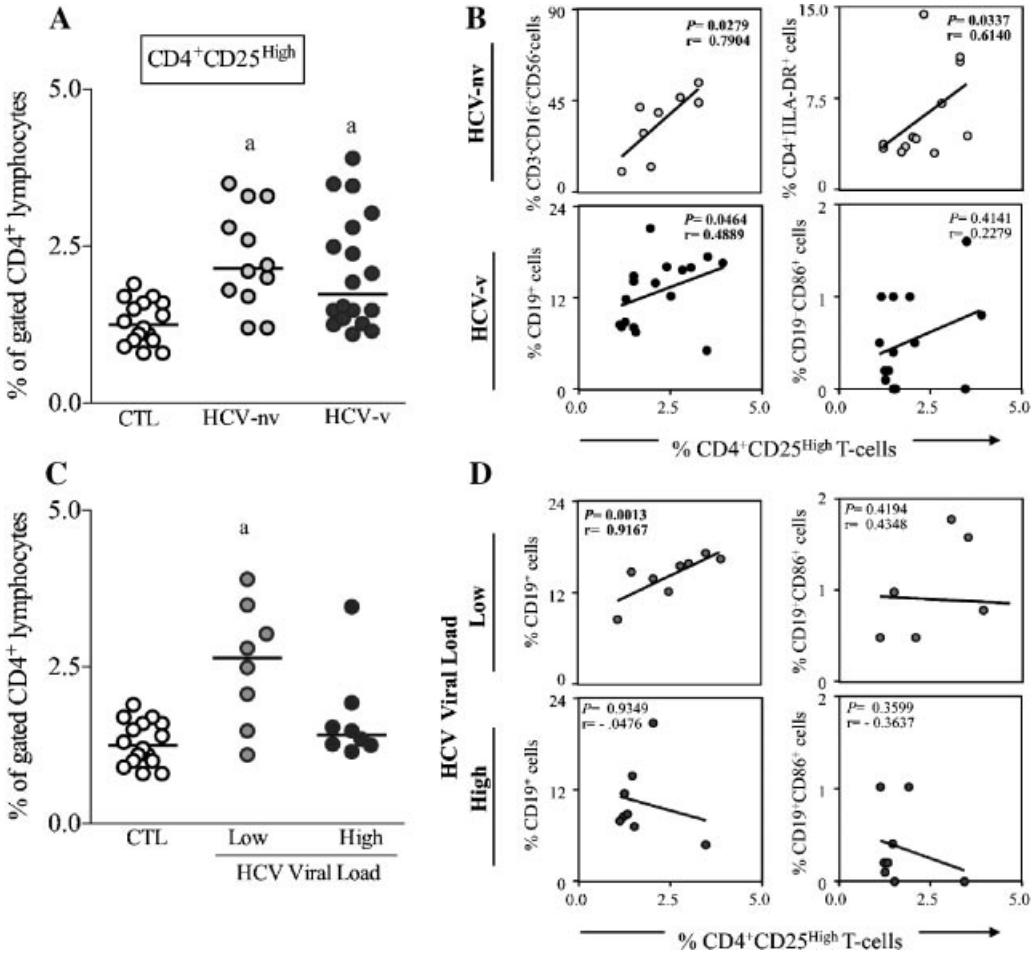

Fig. 6. Analysis of $CD4^+CD25^{High}$ T-cells in the peripheral blood donors from non-viremic (HCV-nv ○ = 12), viremic (HCV-v ● = 19), and healthy control (CTL ○ = 18). Phenotypic studies were performed using a double labeling flow cytometric platform to identify $CD4^+CD25^{High}$ T-cells (**A**) within $CD4^+$ T-cells. The viremic blood donors are shown based on the HCV viral load (**C**): low (◍ = 9) and high (● = 8). Each symbol represents an individual response, and the median is shown. Significant differences at $P < 0.05$ are identified by the letter "a" in comparison with controls. Correlation analysis demonstrated a positive correlation between pre-NK cells, activated $CD4^+$ T-cells, and $CD4^+CD25^{High}$ T-cells in the non-viremic group, and between B-cells and $CD4^+CD25^{High}$ T-cells in the viremic group (**B**). A positive correlation was found between B-cells and $CD4^+CD25^{High}$ T-cells in the viremic subgroup with low HCV viral load (**D**). Correlation indices (r and P-values) are shown in the figure. Statistical differences are highlighted in bold.

besides a lower frequency of mature NK cells ($CD3^-CD16^+CD56^+$), were the hallmark of innate immunity in non-viremic individuals. $CD16^+CD56^-$ pre-NK cells have been considered to be precursors of functional and phenotypically distinct, mature $CD16^+CD56^+$ NK cells. Pre-NK cells have higher proliferative capacity and are better sources of cytokines, while mature NK cells have mainly cytotoxic activities [Sondergaard et al., 2000].

NK cells may be categorized into two distinct subsets according to their surface expression level of CD56. Detailed functional studies have indicated that $CD56^{Dim}$ NK cells are more cytotoxic, while $CD56^{Bright}$ NK cells produce significantly higher levels of cytokines [Cooper et al., 2001; Golden-Mason et al., 2008]. Although, the data on both non-viremic and viremic blood donors (Fig. 2) showed high percentages of $CD56^{Bright}$ NK cells, this was particularly evident in the viremic low viral load subgroup, suggesting that these cells might play an important role in controlling viremia in HCV infection.

NKT cells represent a heterogeneous group of immunoregulatory and effector cells, which express both NK and T-cell markers [Bendelac et al., 1997; Emoto and Kaufmann, 2003]. They may protect against infection through their cytolytic activity by producing cytokines or via stimulation of other cell populations [Godfrey and Kronenberg, 2004]. Interestingly, Huang et al. [1994] proposed that, in humans, these NKT cells subsets might play an important role in eliminating autologous cells in the liver, an important site for the elimination of activated T-cells via apoptosis. On the basis of this report, a three-color platform of immunophenotyping was used to analyze distinct NKT cell subsets, named as follows: NKT1 ($CD3^+/CD16^+/CD56^-$), NKT2 ($CD3^+/CD16^-/CD56^+$), and NKT3 ($CD3^+/CD16^+/CD56^+$). A higher frequency of NKT2 cells ($CD3^+CD16^-CD56^+$) was observed only in the viremic blood donors displaying

a low HCV viral load compared to those with a high load. NKT2 cells produce IL-4, which may inhibit TH1-mediated pathological conditions such as type 1 diabetes [Kadowaki et al., 2001].

In humans and chimpanzees, clearance of HCV in acute infection is followed by a vigorous and multi-specific $CD4^+$ and $CD8^+$ T-cell immune response [Lechner et al., 2000a; Thimme et al., 2001]. The findings revealed increased levels of $CD4^+HLA\text{-}DR^+$ cells particularly in non-viremic individuals compared to viremic individuals, suggesting that these cells might be involved in controlling HCV viremia.

Early responses are essential for the subsequent activation of acquired immunity, composed of antibody secretion by activated B-cells as well as activation of $CD4^+$ and $CD8^+$ cells [Janeway, 2001]. The role of antibodies in protection against HCV, however, has not been elucidated. An increase in $CD19^+$ B-cells and $CD19^+CD86^+$ cells in the viremic blood donors is highlighted, particularly in those with a low viral load. This may indicate that B-cells are important in controlling the viremia levels in this group [Lake-Bakaar et al., 2007].

Recent studies have suggested a major contribution of regulatory $CD4^+$ and $CD8^+$ T-cells in the mechanisms responsible for the dysfunction of HCV-specific T-cells in patients infected chronically [Sugimoto et al., 2003; Accapezzato et al., 2004; Boettler et al., 2005]. In humans, it has been proposed that only the $CD4^+CD25^{High}$ population, comprising ~1–2% of circulating $CD4^+$ T-cells, exhibits regulatory functions [Baecher-Allan et al., 2001].

Despite the fact that, in this study, $CD4^+CD25^{High}$ T-cells were increased in both HCV seropositive subgroups, this subset of cells was increased predominantly in the viremic low viral load subgroup. In addition, a parallel increase in $CD4^+CD25^{High}$ T-cells, pre-NK cells, and activated $CD4^+$ T-cells was observed in non-viremic individuals, while a parallel increase in $CD4^+CD25^{High}$ and B-cells, both hallmarks of viremic group, was found in those samples with a low viral load.

Currently, the best-characterized regulatory T-cells are $CD4^+CD25^+$ T-cells [O'Garra and Vieira, 2004]. Two major populations of $CD4^+CD25^+$ cells can be distinguished: naturally occurring regulatory T-cells (Tregs) and induced Treg cells. Naturally occurring Treg cells consist of $CD4^+$ cells that mature in the thymus into regulatory T-cells. They represent 5–10% of peripheral $CD4^+$ T-cells and express constitutively CD25. Induced Treg cells acquire their suppressive activity during activation in vitro or in vivo and are derived from $CD4^+CD25^+$ T-cells [Bluestone and Abbas, 2003; Vigouroux et al., 2004].

The mechanisms underlying the suppressive functions of Treg cells are not understood completely. Some studies have indicated that direct cell–cell contact is required, while others have suggested that cytokines such as IL-10 and TGF-β may play an important role [Ng et al., 2001; Jonuleit et al., 2002; Shevach, 2002; O'Garra and Vieira, 2004]. An increasing number of studies indicate a potential role for Treg cells in the control of virus-specific T-cell responses during acute and chronic viral infections [Mittrucker and Kaufmann, 2004]. A study by Boettler et al. [2005] suggested that patients with chronic versus resolved HCV infection displayed distinct degrees of Treg suppression of HCV-specific $CD8^+$ T-cell proliferation. In fact, individuals with resolved HCV infection showed lower Treg suppressive activity, despite having the same number of $CD4^+CD25^+$ T-cells, suggesting that the different degrees of suppressive activity of Treg cells may depend on numerous, as yet unknown, factors induced during chronic HCV infection [Boettler et al., 2005].

In contrast to previous studies [Dittmer et al., 2004; Boettler et al., 2005], the findings here suggest that the $CD4^+CD25^{High}$ T-cell-mediated immune response may be beneficial for the control of HCV viremia. The impairment of HCV-specific $CD8^+$ T-cell proliferation and IFN-γ secretion by $CD4^+CD25^{High}$ T-cells in the chronic phase of infection suggests that the role of $CD4^+CD25^{High}$ T-cells is a complex event and that this role may be different during the various stages of HCV infection.

Together, these findings suggest that $CD56^{Bright}$ NK cells, pre-NK cells, and activated $CD4^+$ T-cells, in addition to $CD4^+CD25^{High}$ T-cells, may play an important role in controlling viremia. Elevated $CD56^{Bright}$ NK cells, B-cell responses, and a T-regulated immunological profile appeared to be associated with a low viral load.

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