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Relation	

Lymphocyte subset characterization associated with persistent hepatitis C virus infection and subsequent progression of liver fibrosis

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Abbreviations:

AHS, Adult Health Study; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; Gy, gray; HCV, hepatitis C virus; RERF, Radiation Effects Research Foundation

Abstract

This study aims to deepen understanding of lymphocyte phenotypes related to the course of hepatitis C virus (HCV) infection and progression of liver fibrosis, in a cohort of atomic-bomb survivors. The study subjects comprise three groups: 162 HCV persistently infected, 145 spontaneously cleared, and 3511 uninfected individuals. We found increased percentages of peripheral blood T_H1 and total CD8 T cells and decreased percentages of NK cells in the HCV persistence group, compared with the other two groups, after adjustment for age, gender, and radiation exposure dose. Subsequently, we found that increased T_H1 cell percentages in the HCV persistence group were significantly associated with an accelerated time-course reduction in platelet counts—accelerated progression of liver fibrosis—while T_C1 and NK cell percentages were inversely associated with the progression. This study suggests that T_H1 immunity is enhanced by persistent HCV infection, and that percentages of peripheral T_H1, T_C1, and NK cells may help predict progression of liver fibrosis.

Keywords:

Cohort study; hepatitis C virus; liver fibrosis; lymphocyte subset

Abbreviated title:

Lymphocyte subsets and HCV-infected persons

1. Introduction

HCV infects some 120-170 million people worldwide, and persistent HCV infection is a major cause of liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinomas [1, 2]. It is now widely recognized that both innate and adaptive arms of the host immune system are closely involved in persistent infection, liver injury, and virus clearance [3, 4]. For instance, cytotoxic granule release and cytokine production of NK cells are inhibited by direct binding of HCV envelope protein E2 to CD81 on NK cells, or stabilizing the HLA-E expressions on hepatocytes in HCV-infected patients [5, 6]. However, comprehensive understanding of interactions between HCV and the immune system remains incomplete [3, 4]. Moreover, aging, gender, and several environmental factors such as alcohol drinking, smoking, and ionizing radiation have been reported to influence host immune functions as well as HCV spontaneous clearance [7-9], which may increase the complexity of virus-host interactions. Therefore, a comprehensive characterization of host immunological phenotypes in HCV infection is needed, especially with a cohort-based study design without conceivable selection bias [10]. Nevertheless, few studies along those lines have been carried out. One prospective cohort study (Adult Health Study) of atomic-bomb survivors—a longevity cohort with biennial health examinations—has been conducted at the Radiation Effects Research Foundation (RERF), and the study provides clinicoepidemiological data related to HCV infection and immunological status [11, 12].

Within the cohort study, we conducted a cross-sectional analysis for peripheral blood lymphocyte subsets among HCV persistently-infected, spontaneously-cleared, and uninfected groups, aiming to delineate immunological distinctions among these three groups. We also aimed to identify the lymphocyte subsets that can predict hepatitis progression in HCV-persistent individuals, on the basis of a longitudinal analysis of time-course changes of platelet counts.

2. Materials and methods

2.1. Study population

The Atomic Bomb Casualty Commission, subsequently the RERF, established the Adult Health Study (AHS) cohort in 1958. This cohort study enrolled a total of 23,000 atomic-bomb survivors in Hiroshima and Nagasaki, who biennially received health examinations in outpatient clinics [11]. Hepatitis screening (HBsAg, anti-HBc Ab, anti-HBs Ab, and anti-HCV Ab tests, as well as HCV RNA test if anti-HCV Ab was positive) was conducted among 6,121 AHS participants in 1993 – 1995 [12]. Anti-HCV Ab negative subjects were categorized as the HCV-uninfected group in this study, whereas a "persistence" group was identified by anti-HCV Ab positive with detected HCV RNA, and a "spontaneous clearance" group was identified by anti-HCV Ab positive and undetectable HCV RNA. Subjects with hepatitis B virus surface antigen-positive were excluded from this study. From the 6,121 AHS subjects, lymphocyte subsets in the peripheral blood were then examined in 162 HCV persistence, 145 virus clearance, and 3511 uninfected subjects in 2000 – 2002. Most subjects (N = 120, 74%) in the persistence group (N = 162, including those with cancer history) were confirmed by a second RNA test at least two years after the first RNA test performed in 1993 – 1995. Although the remaining 42 subjects in the group did not undergo the second RNA test, these subjects were confirmed to have developed type C chronic liver disease, based on medical chart review (e.g., treatment history, abdominal sonographic observation, changes in platelet count, zinc sulfate turbidity, AST, and ALT, between 1993 – 1995 and 2000 – 2002) by a hepatologist (one of the authors, WO). Subsequent treatment data of hepatitis C from attending physicians were also taken into account. No subjects in the persistence group underwent IFN therapy in 2000 – 2002.

This study was approved by the RERF Human Investigation Committee, and all subjects gave written informed consent before each examination.

2.2. Assays in hepatitis screening and clinical examinations

In 1993 – 1995, anti-HCV Ab and hepatitis B virus surface antigen were examined using a second-generation passive hemagglutination kit and a reverse passive hemagglutination kit (Dynabott, Tokyo), as described previously [12]. Subjects were diagnosed as having Ab when agglutination was found in a serum diluted 2^5. Qualitative and quantitative detection of HCV RNA was carried out using the Amplicor HCV ver. 2.0 and the Amplicor HCV monitor test ver. 1.0 and/or ver. 2.0 (Roche Diagnostics Systems, Tokyo, Japan).

Platelet count decreases with progression of liver fibrosis, and this marker has widely been used as a reliable diagnostic tool for liver fibrosis/cirrhosis in patients with chronic HCV infection [13-16]. Postulated mechanisms for such platelet reduction include decreased secretion of the hematopoietic growth factor thrombopoietin from the liver and increased destruction of platelets by antiplatelet antibodies [17, 18]. Platelet count was routinely measured in the AHS health examination, and an automatic blood cell counter (Coulter MAXM, Beckman Coulter, Inc. Tokyo, Japan) was used in 2000 – 2002. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GTP), and total cholesterol were also routinely measured, and an autoanalyzer (Hitachi 7180, Hitachi, Ltd., Tokyo, Japan) was used in 2000 – 2002.

2.3. Information on lifestyle/environmental factors and clinical data

Information on alcohol drinking and smoking was obtained from questionnaires at the time of the AHS health examination in 1993 – 1995 and 2000 – 2002, respectively. Body mass index (BMI) was measured at the AHS health examination in 2000 – 2002. Radiation dose was estimated by the DS02 dosimetry system [19], based on the weighted skin dose computed as the gamma dose plus 10 times the neutron dose. No subjects were diagnosed with HIV infection. No subjects underwent organ transplantation or immunosuppressive therapy. Clinical information was obtained at the AHS examination in 2000 – 2002 as well as medical chart review, and classified according to the International Classification of Diseases (ICD) code.

2.4. Lymphocyte subset analysis

Circulating T_H1 and T_H2 cells can be straightforwardly enumerated by flow cytometry, using cell surface markers for chemokine receptor, CXCR3, and prostaglandin D receptor, CRTH2, respectively [20, 21]. CD8 T cells expressing CXCR3, known as T_C1, are also involved in the viral control during HCV infection [22]. We thus focused on T_H1, T_H2, T_C1 and T_C2 cell subsets, as well as total CD4 T and CD8 T, NK, and B cell subsets in relation to HCV infection status.

Analytical flow cytometry was conducted in a FACScan machine (BD Biosciences, San Jose, CA), as described previously [23]. Monoclonal antibodies as specific cell surface markers were purchased from BD Pharmingen (San Diego, CA), unless otherwise noted. CD4 or CD8 T cells were enumerated as PerCP-labeled CD3 positive and PE-CD4 or FITC-CD8 positive cells; CD16 or CD20 cells were enumerated as PerCP-CD3 negative and FITC-CD16 (Beckman Coulter, Brea, CA) or PE-CD20 positive cells. We used CXCR3 as a marker for T_H1 and T_C1 cells [20, 22] and CRTH2 for T_H2 and T_C2 cells [21]. Namely, T_H1 and T_H2 cells were identified with PerCP-CD4, FITC-CXCR3 (R&D Systems, Minneapolis, MN), and biotinylated CRTH2 (kindly provided by Dr. K. Nagata, BML, Kawagoe, Japan) plus PE-streptavidin; T_C1 and T_C2 cells were identified with PerCP-CD8, FITC-CXCR3, and biotinylated CRTH2 plus PE-streptavidin. In every measurement, approximately 20,000 cells were analyzed.

2.5. Statistical analysis

Two sample Wilcoxon or Pearson chi-square tests were performed to compare distributions of age, gender, city, radiation dose (Gy), smoking (packs/day), alcohol drinking (converted to grams of ethanol/day), BMI (kg/m^2), AST (IU/L), ALT (IU/L), γ-GTP (U/L), total cholesterol (mg/dL), and platelet count ($\times 10^4/\mu$L) between all combinations of the three groups.

Since aging and past radiation exposure likely influenced various immunological markers [23], these events were also evaluated in this study. In each study group, the associations of lymphocyte subsets with age (at the time of examination), gender, radiation dose, and city were evaluated based on the multiple regression model [24]:

$$\mathit{log}\ (\mathit{subset\ percentages}\ \text{or}\ \mathit{ratios}) = \alpha + \beta_1 \times \mathit{age} + \beta_2 \times \mathit{gender} + \beta_3 \times \mathit{dose} + \beta_4 \times \mathit{city} + \beta_5 \times \mathit{alcohol} + \beta_6 \times \mathit{smoking} + \beta_7 \times \mathit{BMI} + \beta_8 \times \mathit{autoimmune\ disease} + \beta_9 \times \mathit{allergic\ disease} + \beta_{10} \times \mathit{cancer} + \beta_{11} \times \mathit{other\ non\text{-}cancer\ diseases}$$

where log is the logarithm at base 10, gender = 0 for male and 1 for female, city = 1 for Hiroshima and 2 for Nagasaki. Smoking, alcohol drinking, BMI, autoimmune disease (1 if diagnosed, otherwise 0), allergic disease (1 or 0), cancer (1 or 0), and other non-cancer diseases (i.e., hypergammaglobulinemia and sarcoidosis, 1 or 0) were also used as additional explanatory variables.

We compared lymphocyte subset percentages or ratios between all combinations of the three groups in normal regression analysis with adjustment for age, gender, radiation dose, city, alcohol, smoking, BMI, autoimmune diseases, allergic diseases, and other non-cancer diseases: In the regression analysis, an explanatory variable regarding a group (one group = 0, another group = 1) was used.

Regression analysis was also performed to investigate if there was any association between subset percentages or ratios and time-course changes in platelet counts through the period from 2000 through 2006. Changes in platelet counts were calculated by:

(platelet counts at the last examination – platelet counts at the first examination) / follow-up years.

In a regression analysis, a forward step-wise procedure was used for 8 immunological variables, %CD4, T_H1, T_H2, CD8, T_C1, T_C2, CD16, and CD20. Four variables (%T_H1, T_C1, CD16, and CD20) were consequently selected (significance level to select, $P < 0.2$) to construct a statistical model. All analyses were conducted using Stata software (Stata/SE 9.2 for Windows, StataCorp LP, College Station, TX).

3. Results

3.1. Basic characteristics of study subjects

Table 1 compares characteristics of study subjects in the HCV persistence, clearance, and uninfected groups. The persistence group showed increased levels of blood γ-GTP, and decreased levels of total cholesterol and platelet counts, compared with the other two groups, indicating enhanced liver injury by persistent HCV infection. The proportion of Hiroshima subjects in the persistence or clearance group was higher than that in the uninfected. This is in accordance with the previous study that showed a higher anti-HCV Ab prevalence in Hiroshima atomic-bomb survivors than in Nagasaki survivors [12]. There were no significant differences in radiation dose by HCV infection status.

This study primarily aimed to evaluate immunological alterations associated with HCV infection that might be modulated by age, gender, or past radiation exposure. Therefore, the effects of those factors on lymphocyte subsets were first analyzed and are summarized in Supplemental Tables. In the uninfected group, we found: i) age- and dose-dependent decreases in total CD4 T cell percentages, ii) higher total CD4 T cell percentages in females than in males, iii) no significant effects of age, gender, or radiation dose on total CD8 T cell percentages, iv) increased CD16 (NK) cell percentages with increasing age and higher percentages in males than in females, v) increased T_H1 and T_H2 cell percentages with increasing age and dose, and vi) both T_H1/T_H2 and T_C1/T_C2 cell ratios negatively or positively associated with age and the female gender, respectively, but not with radiation dose (Supplemental Table 1). The persistence and clearance groups also showed similar associations, although most associations were not statistically significant, probably due to smaller numbers of subjects in these groups (Supplemental Tables 2 and 3). In addition to the three factors (age, gender, and radiation), other selected factors such as city, alcohol, smoking, and BMI also influenced various lymphocyte subsets (data not shown), and they were used as confounding variables in adjustments.

3.2. Comparison of lymphocyte subsets among the HCV persistence, clearance, and uninfected groups

The study subjects having cancer history numbered 60, 32, and 698 in the persistence, clearance, and uninfected groups, respectively. We then analyzed the lymphocyte subset alterations associated with HCV infection among subjects who have no history of cancer shown in Table 2, so as to eliminate potential effects of cancer development and/or cancer therapy (Table 3). In addition, basic characteristics were not largely changed by excluding subjects with a history of cancer, but the radiation effects—specifically on T_H1 and T_H2 cells—were no longer seen among subjects with no cancer history (data not shown).

In the persistence group, T_H1 and total CD8 T cell percentages and T_H1/T_H2 ratios were significantly higher than those in the HCV-uninfected group, while total CD4 T and CD16 cell percentages were lower. Similar differences were also seen between the persistence and clearance groups. However, except for total CD8 T cell percentages, no significant differences were observed between the clearance and uninfected groups.

3.3. Relationship between lymphocyte subsets and progression of liver fibrosis in the HCV persistence group

Next, we analyzed the relationship between lymphocyte subsets and platelet counts that had been longitudinally examined at the biennial AHS examination during 2000 − 2006 in the HCV persistence group, excluding subjects with cancer history (Table 4). Average follow-up period was 4.7 years, and average decrement of platelet counts per year was -0.75 ($\times 10^4/\mu L$). We found that increased percentages of T_H1 cells were associated with accelerated time-course reduction in platelet counts—accelerated progression of liver fibrosis (P = 0.027)—while T_C1 and NK cell percentages were inversely associated with the progression (P = 0.027 and 0.058, respectively).

4. Discussion

We investigated immunological alterations associated with HCV infection in a longevity study cohort of atomic-bomb survivors. First, the effects of age, gender, and radiation on total CD4 T, CD8 T, and NK cells were studied in the uninfected group (Supplemental Table 1) and found to be in close agreement with our previous studies [9, 23]. A new finding related to the uninfected group is that percentages of both T_H1 and T_H2 cells increased with increasing radiation dose and age. That result is consistent with our previous findings in an expanded cohort of atomic-bomb survivors, which showed age- and radiation dose-dependent elevations of cytokine levels for both T_H1-related cytokines (IFN-γ and TNF-α) and a T_H2-related cytokine (IL-6) [25].

Second, we found that persistent HCV infection was associated with increases in T_H1/T_H2 cell ratios and CD8 T cell percentages, and a decrease in NK cell percentages (Table 3). Regarding cytokine responses to persistent HCV infection, past reports in diversified patient groups were rather inconsistent: enhanced T_H1 responses [26-29], T_H2 responses [30-32], or both types [33, 34]. Although differing degrees of pathogenesis and/or inflammation among study patient groups may be in part responsible for this discrepancy [34, 35], some potential methodological drawbacks in studies demonstrating T_H2 cytokine predominance were indicated [27]. The present study on lymphocyte subsets suggested enhanced T_H1 immunity in persistent HCV infection, supporting a view that enhanced T_H1 immunity alone is not sufficient to regulate the virus in many cases of HCV infection.

As observed in this study, both decreased and increased percentages of peripheral NK cells and total CD8 T cells, respectively, have been reported in HCV persistence individuals [36-39]. A reduction in NK cells is assumed to be linked to ongoing viremia that may induce continuous proliferation of CD8 T cells. A recent study on murine cytomegalovirus infection showed that NK cells negatively regulated the number and activity of virus-specific CD8 T cells as well as CD4 T cells that played a critical role in limiting viral persistence; lack of NK cell activation resulted in increased numbers of CD8 T and CD4 T cells along with enhanced effector functions through antigen-presentation by viral infected APCs [40]. Such functional interplay among NK cells, APCs, and CD8 T cells may be common features in virally infected hosts. It is also plausible that reduced NK cells of individuals may in part reflect their weakened natural immunity upon HCV infection, preferentially leading to failure of HCV-infected cell clearance [41].

Finally, our follow-up survey of the HCV persistence group showed that increased T_H1 cell percentages were associated with accelerated progression of liver fibrosis, while T_C1 and NK cell percentages were inversely associated with the progression (Table 4). In accordance with preceding studies [26, 28, 42], this study demonstrates that T_H1-immunity plays a vital role in HCV-related fibrosis progression. In the liver as well as peripheral blood of individuals with chronic HCV infection, T_H1 cells may enhance CTL response and macrophage activation by producing cytokines such as IL-2, IFN-γ, and TNF-α, thereby facilitating the necroinflammatory process of hepatitis C [26, 42]. On the other hand, increased T_C1 cell percentages in total CD8 T cells were associated with slower progression of fibrosis—new findings in this study. T_C1 cells express a chemokine receptor, CXCR3, which is required for migration to the HCV-infected liver [22], and we inferred that the increased T_C1 fraction includes HCV-specific CD8 T cells. Several studies have shown relationships between higher numbers of circulating as well as intrahepatic HCV-specific CD8 T cells and lesser degrees of liver fibrosis during chronic HCV infection [43-46]: One plausible explanation is that HCV-specific CD8 T cells might control the virus without exerting cytotoxic effects on hepatocytes [46]. Alternatively, a population of CD8 T cells secreting an anti-fibrotic cytokine, IL-10, may be implicated in attenuation of hepatocyte killing and protection against liver injury [45, 47].

A limitation of this study is that we examined clinical and immunological data only from peripheral blood. Intrahepatic lymphocytes are assumed to have features discrete from those in peripheral blood [48]. Also, our comparison of lymphocyte subsets among study groups was cross-sectional, making it difficult to identify immunological factors responsible for persistent HCV infection.

In conclusion, this study identified immunological characteristics associated with HCV infection in a Japanese population and also indicated that peripheral T_H1, T_C1, and NK cell subsets will be useful for predicting progression of hepatitis in persistently HCV-infected patients, and consequent development of hepatocellular carcinomas.

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References

[1] Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 2005;5:558-67.

[2] Fung J, Lai CL, Yuen MF. Hepatitis B and C virus-related carcinogenesis. Clin Microbiol Infect 2009;15:964-70.

[3] Post J, Ratnarajah S, Lloyd AR. Immunological determinants of the outcomes from primary hepatitis C infection. Cell Mol Life Sci 2009;66:733-56.

[4] Sklan EH, Charuworn P, Pang PS, Glenn JS. Mechanisms of HCV survival in the host. Nat Rev Gastroenterol Hepatol 2009;6:217-27.

[5] Crotta S, Stilla A, Wack A, D'Andrea A, Nuti S, D'Oro U, et al. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. J Exp Med 2002;195:35-41.

[6] Nattermann J, Nischalke HD, Hofmeister V, Ahlenstiel G, Zimmermann H, Leifeld L, et al. The HLA-A2 restricted T cell epitope HCV core 35-44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. Am J Pathol 2005;166:443-53.

[7] Piasecki BA, Lewis JD, Reddy KR, Bellamy SL, Porter SB, Weinrieb RM, et al. Influence of alcohol use, race, and viral coinfections on spontaneous HCV clearance in a US veteran population. Hepatology 2004;40:892-9.

[8] Stampfli MR, Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. Nat Rev Immunol 2009;9:377-84.

[9] Kusunoki Y, Hayashi T. Long-lasting alterations of the immune system by ionizing radiation exposure: implications for disease development among atomic bomb survivors. Int J Radiat Biol 2008;84:1-14.

[10] Ellenberg JH, Nelson KB. Sample selection and the natural history of disease. Studies of febrile seizures. JAMA 1980;243:1337-40.

[11] Beebe GW, Fujisawa H, Yamasaki M. Adult Health Study Reference Papers, A: Selection of the Sample, and B: Characteristics of the Sample. Technical Report 10-60. Hiroshima: Atomic Bomb Casualty Comission; 1960.

[12] Fujiwara S, Kusumi S, Cologne J, Akahoshi M, Kodama K, Yoshizawa H. Prevalence of anti-hepatitis C virus antibody and chronic liver disease among atomic bomb survivors. Radiat Res 2000;154:12-9.

[13] Ono E, Shiratori Y, Okudaira T, Imamura M, Teratani T, Kanai F, et al. Platelet count reflects stage of chronic hepatitis C. Hepatol Res 1999;15:192-200.

[14] Pohl A, Behling C, Oliver D, Kilani M, Monson P, Hassanein T. Serum aminotransferase levels and platelet counts as predictors of degree of fibrosis in chronic hepatitis C virus infection. Am J Gastroenterol 2001;96:3142-6.

[15] Coverdale SA, Samarasinghe DA, Lin R, Kench J, Byth K, Khan MH, et al. Changes in antipyrine clearance and platelet count, but not conventional liver tests, correlate with fibrotic change in chronic hepatitis C: value for predicting fibrotic progression. Am J Gastroenterol 2003;98:1384-90.

[16] Moriyama M, Matsumura H, Aoki H, Shimizu T, Nakai K, Saito T, et al. Long-term outcome, with monitoring of platelet counts, in patients with chronic hepatitis C and liver cirrhosis after interferon therapy. Intervirology 2003;46:296-307.

[17] Kawasaki T, Takeshita A, Souda K, Kobayashi Y, Kikuyama M, Suzuki F, et al. Serum thrombopoietin levels in patients with chronic hepatitis and liver cirrhosis. Am J Gastroenterol 1999;94:1918-22.

[18] Olariu M, Olariu C, Olteanu D. Thrombocytopenia in chronic hepatitis C. J Gastrointestin Liver Dis 2010;19:381-5.

[19] Cullings HM, Fujita S, Funamoto S, Grant EJ, Kerr GD, Preston DL. Dose estimation for atomic bomb survivor studies: its evolution and present status. Radiat Res 2006;166:219-54.

[20] Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 1998;187:875-83.

[21] Cosmi L, Annunziato F, Galli MIG, Maggi RME, Nagata K, Romagnani S. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. Eur J Immunol 2000;30:2972-9.

[22] Larrubia JR, Calvino M, Benito S, Sanz-de-Villalobos E, Perna C, Perez-Hornedo J, et al. The role of CCR5/CXCR3 expressing CD8+ cells in liver damage and viral control during persistent hepatitis C virus infection. J Hepatol 2007;47:632-41.

[23] Yamaoka M, Kusunoki Y, Kasagi F, Hayashi T, Nakachi K, Kyoizumi S. Decreases in percentages of naive CD4 and CD8 T cells and increases in percentages of memory CD8 T-cell subsets in the peripheral blood lymphocyte populations of A-bomb survivors. Radiat Res 2004;161:290-8.

[24] Armitage P, Berry G, Matthews JNS. Statistical methods in medical research. 4th ed. Oxford: Blackwell Scientific; 2002.

[25] Hayashi T, Morishita Y, Kubo Y, Kusunoki Y, Hayashi I, Kasagi F, et al. Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors. Am J Med 2005;118:83-6.

[26] Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW. Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology 1996;24:759-65.

[27] Bergamini A, Bolacchi F, Cerasari G, Carvelli C, Faggioli E, Cepparulo M, et al. Lack of evidence for the Th2 predominance in patients with chronic hepatitis C. Clin Exp Immunol 2001;123:451-8.

[28] Sobue S, Nomura T, Ishikawa T, Ito S, Saso K, Ohara H, et al. Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. J Gastroenterol 2001;36:544-51.

[29] Gigi E, Raptopoulou-Gigi M, Kalogeridis A, Masiou S, Orphanou E, Vrettou E, et al. Cytokine mRNA expression in hepatitis C virus infection: TH1 predominance in patients with chronic hepatitis C and TH1-TH2 cytokine profile in subjects with self-limited disease. J Viral Hepat 2008;15:145-54.

[30] Reiser M, Marousis CG, Nelson DR, Lauer G, Gonzalez-Peralta RP, Davis GL, et al. Serum interleukin 4 and interleukin 10 levels in patients with chronic hepatitis C virus infection. J Hepatol 1997;26:471-8.

[31] Fan XG, Liu WE, Li CZ, Wang ZC, Luo LX, Tan DM, et al. Circulating Th1 and Th2 cytokines in patients with hepatitis C virus infection. Mediators Inflamm 1998;7:295-7.

[32] Abayli B, Canataroglu A, Akkiz H. Serum profile of T helper 1 and T helper 2 cytokines in patients with chronic hepatitis C virus infection. Turk J Gastroenterol 2003;14:7-11.

[33] Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. Hepatology 1996;24:6-9.

[34] Kakumu S, Okumura A, Ishikawa T, Yano M, Enomoto A, Nishimura H, et al. Serum levels of IL-10, IL-15 and soluble tumour necrosis factor-alpha (TNF-alpha) receptors in type C chronic liver disease. Clin Exp Immunol 1997;109:458-63.

[35] Anthony DD, Post AB, Valdez H, Peterson DL, Murphy M, Heeger PS. ELISPOT analysis of hepatitis C virus protein-specific IFN-gamma-producing peripheral blood lymphocytes in infected humans with and without cirrhosis. Clin Immunol 2001;99:232-40.

[36] Chan TM, Ho SK, Lai CL, Cheng IK, Lai KN. Lymphocyte subsets in renal allograft recipients with chronic hepatitis C virus infection. Nephrol Dial Transplant 1999;14:717-22.

[37] Chang KM, Thimme R, Melpolder JJ, Oldach D, Pemberton J, Moorhead-Loudis J, et al. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. Hepatology 2001;33:267-76.

[38] Par G, Rukavina D, Podack ER, Horanyi M, Szekeres-Bartho J, Hegedus G, et al. Decrease in CD3-negative-CD8dim(+) and Vdelta2/Vgamma9 TcR+ peripheral blood lymphocyte counts, low perforin expression and the impairment of natural killer cell activity is associated with chronic hepatitis C virus infection. J Hepatol 2002;37:514-22.

[39] Morishima C, Paschal DM, Wang CC, Yoshihara CS, Wood BL, Yeo AE, et al. Decreased NK cell frequency in chronic hepatitis C does not affect ex vivo cytolytic killing. Hepatology 2006;43:573-80.

[40] Andrews DM, Estcourt MJ, Andoniou CE, Wikstrom ME, Khong A, Voigt V, et al. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J Exp Med 2010;207:1333-43.

[41] Golden-Mason L, Cox AL, Randall JA, Cheng L, Rosen HR. Increased natural killer cell cytotoxicity and NKp30 expression protects against hepatitis C virus infection in high-risk individuals and inhibits replication in vitro. Hepatology 2010;52:1581-9.

[42] Moura AS, Carmo RA, Teixeira AL, Leite VH, Rocha MO. Soluble inflammatory markers as predictors of liver histological changes in patients with chronic hepatitis C virus infection. Eur J Clin Microbiol Infect Dis 2010;29:1153-61.

[43] Sreenarasimhaiah J, Jaramillo A, Crippin J, Lisker-Melman M, Chapman WC, Mohanakumar T. Lack of optimal T-cell reactivity against the hepatitis C virus is associated with the development of fibrosis/cirrhosis during chronic hepatitis. Hum Immunol 2003;64:224-30.

[44] Cardoso EM, Duarte MA, Ribeiro E, Rodrigues P, Hultcrantz R, Sampaio P, et al. A study of some hepatic immunological markers, iron load and virus genotype in chronic hepatitis C. J Hepatol 2004;41:319-26.

[45] Abel M, Sene D, Pol S, Bourliere M, Poynard T, Charlotte F, et al. Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. Hepatology 2006;44:1607-16.

[46] Bonilla N, Barget N, Andrieu M, Roulot D, Letoumelin P, Grando V, et al. Interferon gamma-secreting HCV-specific CD8+ T cells in the liver of patients with chronic C hepatitis: relation to liver fibrosis--ANRS HC EP07 study. J Viral Hepat 2006;13:474-81.

[47] Accapezzato D, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, et al. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest 2004;113:963-72.

[48] Grabowska AM, Lechner F, Klenerman P, Tighe PJ, Ryder S, Ball JK, et al. Direct ex vivo comparison of the breadth and specificity of the T cells in the liver and peripheral blood of patients with chronic HCV infection. Eur J Immunol 2001;31:2388-94.

Table 1

Characteristics of the study subjects

	Persistence (anti-HCV + /HCV RNA +)	Clearance (anti-HCV + /HCV RNA -)	Uninfected (anti-HCV -)	Persistence vs. Clearance	Persistence vs. Uninfected	Clearance vs. Uninfected
	N =162	N = 145	N = 3511	P value[a]	P value[a]	P value[a]
Age[b]	71.7 (60.8-83.0)	72.3 (64.8-88.0)	72.2 (58.4-87.2)	0.041	0.79	0.029
Gender[c]						
Male	57 (35.2)	48 (33.1)	1078 (30.7)	0.70	0.23	0.54
Female	105 (64.8)	97 (66.9)	2433 (69.3)			
City[c]						
Hiroshima	112 (69.1)	96 (66.2)	2034 (57.9)	0.58	0.005	0.048
Nagasaki	50 (30.9)	49 (33.8)	1477 (42.1)			
Radiation dose (Gy)[b]	0.147 (0-2.658)	0.071 (0-1.890)	0.096 (0-2.032)	0.26	0.47	0.39
Smoking (pack/day)[b]	0 (0-1.0)	0 (0-1.0)	0 (0-1.0)	0.80	0.087	0.18
Alcohol drinking (gram/day)[b]	0 (0-85.0)[d]	0 (0-103.0)[d]	0 (0-69.8)[d]	0.35	0.57	0.062
BMI (kg/m^2)[b]	22.4 (16.7-28.5)	22.6 (17.3-29.6)	22.7 (17.6-28.7)	0.46	0.084	0.58
AST (IU/L)[b]	21 (15-51)	21 (14-35)	22 (15-43)	0.15	0.85	0.081
ALT (IU/L)[b]	17 (9-46)	18 (9-42)	17 (9-44)	0.87	0.99	0.84
γ-GTP (U/L)[b]	28.5 (12.5-131.5)	25 (12-127)	24 (11-111)	0.10	< 0.001	0.29
Total cholesterol (mg/dL)[b]	168.5 (117-234)	206 (142-264)	208 (154-266)	< 0.001	< 0.001	0.41
Platelet count ($\times 10^4/\mu\text{L}$)[b]	17.3 (7.2-28.2)	22.0 (14.3-30.8)	22.9 (14.5-33.7)	< 0.001	< 0.001	0.031

[a] Two-sample Wilcoxon rank-sum test, or Pearson chi-square test for gender and city.

[b] Median (5-95% percentiles).

[c] Number (%).

[d] The percentages of never-drinkers were 59.3, 53.8, and 60.6 in the 3 groups, respectively.

Table 2

Characteristics of the study subjects with no cancer history

	Persistence (anti-HCV + /HCV RNA +)	Clearance (anti-HCV + /HCV RNA -)	Uninfected (anti-HCV -)	Persistence vs. Clearance	Persistence vs. Uninfected	Clearance vs. Uninfected
	N =102	N = 113	N = 2813	P value[a]	P value[a]	P value[a]
Age[b]	71.5 (61.3-82.7)	72.3 (62.4-90.8)	72.0 (58.1-87.3)	0.026	0.64	0.021
Gender[c]						
Male	36 (35.3)	38 (33.6)	832 (29.6)	0.80	0.22	0.36
Female	66 (64.7)	75 (66.4)	1981 (70.4)			
City[c]						
Hiroshima	70 (68.6)	77 (68.1)	1613 (57.3)	0.94	0.023	0.023
Nagasaki	32 (31.4)	36 (31.9)	1200 (42.7)			
Radiation dose (Gy)[b]	0.031 (0-1.862)	0.056 (0-1.890)	0.072 (0-1.878)	0.84	0.68	0.49
Smoking (pack/day)[b]	0 (0-1.0)	0 (0-1.0)	0 (0-1.0)	0.67	0.070	0.18
Alcohol drinking (gram/day)[b]	0 (0-90)[d]	0 (0-105.8)[d]	0 (0-69.8)[d]	0.067	0.76	0.014
BMI (kg/m^2)[b]	22.6 (16.7-28.5)	22.6 (17.3-29.6)	22.9 (17.7-28.8)	0.49	0.097	0.55
AST (IU/L)[b]	22 (15-50)	21 (13-37)	22 (15-42)	0.12	0.34	0.26
ALT (IU/L)[b]	18 (9-39)	17 (9-48)	17 (9-44)	0.57	0.69	0.69
γ-GTP (U/L)[b]	26 (12-100)	27 (12-127)	23 (11-106)	0.99	0.19	0.18
Total cholesterol (mg/dL)[b]	175 (124-243)	211 (136-272)	209 (157-266)	< 0.001	< 0.001	0.46
Platelet count (×10^4/μL)[b]	19.3 (10.3-32.0)	21.7 (14.2-30.8)	23.0 (14.8-33.7)	< 0.001	< 0.001	0.006

[a] Two-sample Wilcoxon rank-sum test, or Pearson chi-square test for gender and city.

[b] Median (5-95% percentiles).

[c] Number (%).

[d] The percentages of never-drinkers were 64.7, 50.4, and 61.1 in the 3 groups, respectively.

Table 3

Comparisons of peripheral lymphocyte subsets among subjects with no cancer history

	Persistence	Clearance	Uninfected	Persistence vs. Clearance	Persistence vs. Uninfected	Clearance vs. Uninfected
	N = 102	N = 113	N = 2813	P value[a]	P value[a]	P value[a]
CD4 (%)[b]	40.8 (8.9)	42.2 (9.0)	43.0 (8.9)	0.32	0.007	0.46
T_H1 (%)[b]	35.2 (9.6)	27.1 (8.7)	26.0 (8.9)	< 0.001	< 0.001	0.23
T_H2 (%)[b]	1.55 (0.88)	1.74 (1.03)	1.79 (1.10)	0.54	0.11	0.65
T_H1/T_H2[b]	30.7 (20.6)	20.8 (12.8)	20.8 (23.0)	< 0.001	< 0.001	0.27
CD8 (%)[b]	23.4 (9.6)	20.9 (7.9)	19.0 (7.8)	0.20	< 0.001	0.040
T_C1 (%)[b]	42.3 (14.7)	38.8 (15.2)	39.5 (14.6)	0.25	0.17	0.68
T_C2 (%)[b]	2.78 (3.72)	2.87 (4.03)	3.35 (5.09)	0.34	0.68	0.40
T_C1/T_C2[b]	42.8 (45.8)	50.9 (78.2)	51.1 (91.4)	0.66	0.38	0.51
CD4/CD8[b]	2.08 (1.02)	2.42 (1.35)	2.75 (1.57)	0.15	< 0.001	0.054
CD16 (%)[b]	14.0 (9.4)	17.0 (8.8)	17.1 (9.4)	0.035	< 0.001	0.82
CD20 (%)[b]	14.5 (7.6)	13.5 (5.3)	13.9 (6.1)	0.95	0.44	0.60

[a] Test of difference of logarithmic values between two groups using normal regression analysis with adjustment for age, gender, city, radiation dose, alcohol, smoking, BMI, autoimmune disease, allergic disease, and other non-cancer diseases.

[b] Mean (SD).

Table 4

Regresson analysis of decrements (per year) in platelet counts among HCV persistence subjects with no cancer history (N = 96)

	Unadjusted		Adjusted[a]		Adjusted[b]	
Explanatory variables	coefficient	P value	coefficient	P value	coefficient	P value
Age (+10 yrs)	-0.26	0.33	—	—	—	—
Gender (female vs. male)	0.43	0.21	—	—	—	—
Radiation dose (Gy)	0.28	0.26	—	—	—	—
City (Nagasaki vs. Hiroshima)	0.00	0.99	—	—	—	—
Log CD4	-0.25	0.86	1.11	0.50	—	—
Log T_H1	-1.90	0.13	-2.38	0.086	-3.19	0.027
Log T_H2	-0.53	0.41	-0.34	0.65	—	—
Log T_H1/T_H2	0.03	0.97	-0.31	0.67	—	—
Log CD8	-1.72	0.060	-2.63	0.011	—	—
Log T_C1	1.66	0.068	2.05	0.041	2.36	0.027
Log T_C2	-0.30	0.37	0.23	0.57	—	—
Log T_C1/T_C2	0.51	0.12	0.10	0.81	—	—
Log CD4/CD8	1.04	0.16	2.03	0.015	—	—
Log CD16	0.73	0.21	1.13	0.062	1.18	0.058
Log CD20	1.10	0.11	1.04	0.15	1.03	0.17

[a] Regression model: $\textit{decrements in platelet counts} = \alpha + \beta_1 \times \textit{log (lymphocyte subset)} + \beta_2 \times \textit{age} + \beta_3 \times \textit{gender} + \beta_4 \times \textit{dose} + \beta_5 \times \textit{city} + \beta_6 \times \textit{alcohol} + \beta_7 \times \textit{smoking} + \beta_8 \times \textit{BMI} + \beta_9 \times \textit{autoimmune disease} + \beta_{10} \times \textit{allergic disease} + \beta_{11} \times \textit{other non-cancer diseases.}$

[b] Forward step-wise procedure (P < 0.2) was used for 8 lymphocyte variables (CD4, T_H1, T_H2, CD8, T_C1, T_C2, CD16, and CD20). Selected 4 variables (T_H1, T_C1, CD16, and CD20) were used in the regression analysis with 10 explanatory variables in footnote a.