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Expansion of $CD4^+CD25^+FoxP3^+$ Regulatory T Cells in Chronic Hepatitis C Virus Infection

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ABSTRACT

Background: Regulatory T cells (Tregs) have been involved in impaired immunity and may have a pivotal role in persistence of viral infections. **Objective:** To develop a simple and reliable in-house three color flow cytometery of peripheral blood to understand the role of HCV infection in the increase of Tregs. **Methods:** The level of naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T cells (nTregs) in 20 chronically infected with hepatitis C virus (HCV) patients was compared to those of 15 healthy individuals by flowcytometry. In a different approach we performed permeabilization and intracellular staining before surface staining which allows the preservation of the surface molecules in the combined detection process and results in the normal frequency of nTregs in blood. **Results:** Using the optimized method, it was shown that a significantly higher proportion of nTregs in the total $CD4^+$ T cell population was seen in the peripheral blood of chronic HCV patients ($0.83 \pm 0.21\%$, $p=0.05$) as compared to controls (0.26 ± 0.1, $p=0.05$). **Conclusions:** In accordance with other studies, we showed that HCV infection induces a dramatic increase in Tregs, which might contribute to the immune response failure during HCV infection.

Keywords: Hepatitis C Virus, Flowcytometry Method, Natural Regulatory T Cells

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INTRODUCTION

Hepatitis C virus (HCV) is one of the most important causes of persistent infection in humans. This virus is associated with a high rate of chronic hepatitis and a highly severe liver destruction (1,2). The pathogenesis of HCV has been associated with the interaction between host immune response and viral factors (3). Although it is now firmly established that an impaired specific effector T cell response is the main cause of the development of chronic HCV infection, little is known about the host factors contributing to an insufficient T cell response (4). In recent years, among several mechanisms that have been suggested to explain this abnormal effector immune response, regulatory T cells (Tregs) have been subjected to more attention (2,5). Tregs are a subset of circulating $CD4^+$ T cells with suppressive properties (6).

Regulatory T cells are a heterogeneous group of T cells which include naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T (nTreg) cells (7) that develop during the process of T cell maturation in the thymus, induced Treg (iTreg) cells which are obtained after oral antigen administration (Th3), the T cells that produce imunosuppressive cytokines such as IL-10 (Tr1 cells) (8), subsets of $CD8^+$ T cells, double negative T cells and γδ T cells (9). The minor population of $CD4^+$ T cells expresses a chain of IL-2 receptor (CD25) on the surface (10).

Naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T cells (nTregs) (6) develop during the process of T cell maturation in the thymus. The nTregs were recognized by their constitutive expression of CD4, CD25 and expression of forkhead-family transcription factor (FoxP3) (10). FoxP3 is a transcriptional repressor (11) and is expressed specifically by T cells with regulatory functions (12). The literature suggests that FoxP3 can represent the best marker for regulatory T cell activity (13). However, Wang et al. observed that FoxP3 is expressed in a high percentage of activated T cells after in vitro stimulation of human $CD4^+CD25^-$ cells. The expression of endogenous FoxP3 in humans might not be sufficient to identify Treg cells after in vitro stimulation (14), although it is a reliable marker for detection of Tregs in unstimulated PBMCs (1,12).

Although Tregs play a critical role in maintaining a balance between preventing immunopathology and allowing the immune response to clear infections (15), some viruses can evade the immune response by shifting toward Tregs developement (1,13,16). In HCV infection, elevation of Tregs may contribute to persistent HCV infection by suppressing HCV specific T-cell responses (2,13,17-21). Different groups have analyzed levels of Tregs in HCV infection (1,5,2,19,22,23). Not in all, but in the majority of reports, an increased frequency of CD4+CD25+ Tregs in chronically HCV-infected patients has been found (19, 24, 25), supporting the contribution of Tregs to the impaired virus-specific T-cell responses in chronic HCV infection. In more recent controversial results, no increase in the level of Tregs in chronically HCV infected patients was obtained (1,23), however, this result was not supported by Yoshizawa et al. (19). In the light of these considerations, the aim of the present study was to investigate the frequency of nTregs in HCV-infected patients. Currently, there are many protocols and kits for simultaneous detection of intracellular and cell surface antigens of Tregs. Speed, efficiency and cost with a minimum loss of surface antigens during cell permeabilization are important considerations in all protocols. We describe here an efficient and cheap

in-house protocol for the identification of nTregs in human blood. Tregs were detected as $CD4^+$, $CD25^+$ and $FoxP3^+$ cell population in a complex mixture of peripheral blood mononuclear cells (PBMCs) and the frequency of Tregs was examined with staining both intracellular and cell surface antigens, while preserving cell morphology.

MATERIALS AND METHODS

Study Population. The study was approved by institutional ethics committee and informed consent was obtained from all patients. A descriptive study was conducted on individuals attending Digestive Disease Research Center (DDRC) of Shariati Hospital (Tehran, Iran). The study population included 20 HCV infected patients and 15 healthy controls. HCV patients were IFN-naive and were checked for HCV, human immunodeficiency virus (HIV) and hepatitis B virus (HBV) by commercially available ELISA kits (Organon Technica, Turnhout, Belgium) and were histologically classified as chronic active hepatitis with positive anti HCV-antibodies. Table 1 summarizes the main characteristics of the study population.

Antibodies Used in Flowcytometry. The following conjugated antibodies (Abs) from BD Pharmingen (USA) were used in flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Cat. no.555346), phycoerythrin (PE)-conjugated anti-FoxP3 (Cat no. 560046) and phycoerythrin (PE)/Cy7-conjugated anti-CD25 (Cat no. 335824).

Mononuclear Cell Separation. PBMCs were separated from fresh ethylenediamine tetraacetic acid (EDTA)-anti-coagulated blood by density gradient centrifugation using Ficoll-Hypaque. PBMCs were washed and resuspended in RPMI-1640 (Gibco, UK) containing 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (complete medium).

Optimizing the Antibody Dilution. The optimal concentration of each antibody was determined using a series of dilutions in a titration experiment to determine the optimal antibody dilution according to the previously reported frequency for each marker.

Mono-Color Flowcytometry for Surface Markers, CD4 and CD25. After isolation of PBMCs, 2×10^5 washed cells were fixed with 4% paraformaldehyde (PFA) for improvement in consequence intracellular staining, and rinsed in 2 ml PBS containing 1 mM EDTA and 1% FBS (washing buffer). Mouse serum was used as a blocking solution followed by washing. Anti-CD4 antibody was applied at optimum dilution for 30 minutes in the dark at 4°C. The cells were then washed and resuspended in 2% PFA and analyzed on a cytometer. In a similar experiment, the optimal dilution of antibody coupled to PE-CY7 was used for the detection of CD25 positive lymphocyte cells.

Dual-Color Flowcytometry for CD4 and FoxP3. Cells were fixed, permeabilized with 0.1% Triton X-100 (Sigma, UK) and stained with appropriate dilution of anti-FoxP3 antibody for 45 min at 4°C in the dark. After washing, the cells were incubated with a optimum dilution of monoclonal anti-CD4 antibody for 30 minutes, washed and resuspended in 2% PFA.

Table 1. The demographic and clinical information as well as percentages of nTregs are shown for 20 chronic HCV subjects and 15 normal ones. A significantly higher proportion of Tregs in the total CD4+ T cell population was seen in the peripheral blood of chronic HCV patients (0.83 ± 0.21%) as compared to the healthy controls (0.26 ± 0.11%) (p= 0.05).

Pateint[a]	Age (years)	Sex[b]	Genotype	Viral load (copies/ml×10^3)	% Tregs
CHC[c]-1	32	f	1a	731100	0.57
CHC-2	25	f	1a	1821000	0.89
CHC-3	35	m	1a	33930	1.06
CHC-4	59	m	1a	6520000	1.09
CHC-5	30	m	1a	2380000	0.98
CHC-6	55	m	1a	22560000	1.22
CHC-7	30	m	1a	2845	0.49
CHC-8	47	m	1a	156700	0.81
CHC-9	55	f	1b	574200	0.7
CHC-10	56	m	1a	6925000	1.01
CHC-11	46	f	1a	901000	0.9
CHC-12	59	m	1a	845800	0.89
CHC-13	27	m	1a	6832000	0.53
CHC-14	58	f	1b	919300	0.78
CHC-15	36	m	1a	350600	0.56
CH-16	47	m	1a	584000	0.51
CHC-17	33	m	1a	4126000	0.83
CHC-18	51	m	1a	23652376	0.87
CHC-19	30	m	1a	701234	0.97
CHC-20	39	f	1a	6421234	0.95
Normal[d]-1	32	f	n.a.[e]	n.a.	0.22
Normal-2	25	f	n.a.	n.a.	0.1
Normal-3	31	m	n.a.	n.a.	0.34
Normal-4	45	f	n.a.	n.a.	0.15
Normal-5	30	m	n.a.	n.a.	0.22
Normal-6	52	m	n.a.	n.a.	0.44
Normal-7	30	m	n.a.	n.a.	0.23
Normal-8	47	f	n.a.	n.a.	0.25
Normal-9	29	f	n.a.	n.a.	0.41
Normal-10	33	m	n.a.	n.a.	0.12
Normal-11	46	f	n.a.	n.a.	0.44
Normal-12	31	m	n.a.	n.a.	0.33
Normal-13	27	m	n.a.	n.a.	0.14
Normal-14	29	m	n.a.	n.a.	0.3
Normal-15	36	f	n.a.	n.a.	0.22

[a] None of the chronic patients had been treated with IFN-α
[b] f, Female; m, male
[c] Chronic hepatitis C patients
[d] Healthy control
[e] not applicable

Use of Three-Color Flowcytometry for Detection of Circulating nTreg Cells. To determine the frequency of peripheral blood nTreg cells, the intracellular staining was done and the stained cells were incubated with surface antibodies as described. Isotype-matched negative control antibodies were used to assess the level of non-specific binding, and also used as a guide for setting markers to delineate positive and negative populations. Three-colour acquisition was performed on FACSCalibur (Becton Dickinson).
Statistical Analysis. Kruscal-Wallis test was used to compare the mean values of nTregs in HCV infected pateints with the control group. Analysis has been done in SPSS 16 software and a p-value of 0.05 is considered statistically significant.

RESULTS

Surface Cellular Detection of Antigens. PBMCs from HCV exposed individuals and control groups were isolated to establish a suitable procedure for the detection of nTregs. The optimal antibody concentrations for detection of CD4 and CD25 markers were determined experimentally using serial dilutions of each antibody in a titration experiment. Optimal working dilutions of anti-CD4 and anti-CD25 antibodies obtained were 1 µl of stock solution in a volume of 20 µl for anti-CD4 antibody and 1 µl of stock solution for anti-CD25 antibody. The average percent of CD4 and CD25 positive cells in PBMCs were showed to be 50 and 1-2, respectively.
Strategy for the Combined Detection of Surface and Intracellular Antigens on PBMCs. To perform an accurate analysis of nTregs by flow cytometry, a combined detection of both surface and intracellular antigens was proposed. To access mAbs to cytoplasmic molecules, membrane permeabilization step was required. Triton X-100 is usually used in the process of permeabilization for intracellular staining. To study the effect of triton permeabilization on the loss of surface molecules, we followed two staining procedures. In the first procedure performed according to the commercial protocols, the permeabilization and FoxP3 staining were done after surface staining, which resulted in significantly decreased CD4 markers on lymphocytes (Figure 1a).

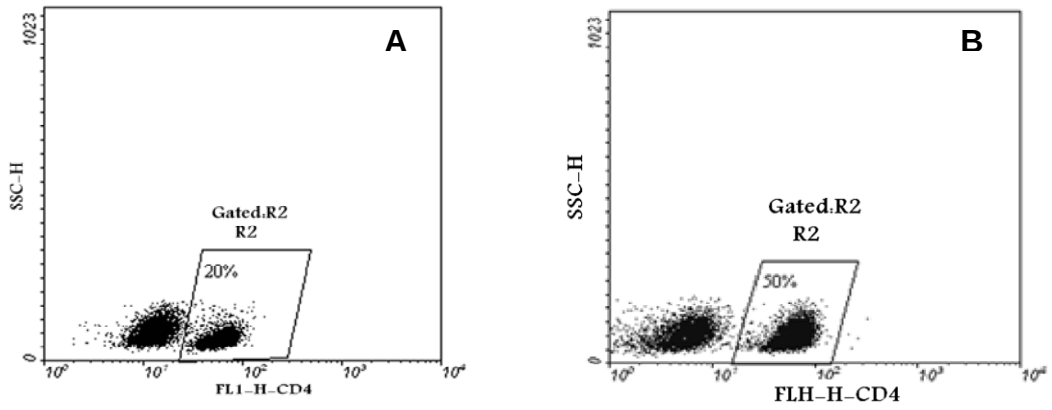

Figure1. Detection of CD4 positive cells in two different methods of intercellular staining (for details, see results). A) PBMCs from healthy donor were fixed and surface staining with anti-CD4 mAbs was performed before permeabilization which caused a sharp decrease in CD4 frequency. B) Surface staining with anti-CD4 was done after permeabilisation of PBMCs in which percentages of CD4 markers was not changed.

In this protocol the average percent of CD4 positive cells decreased from 45 ± 10 to 20 ± 3. In the next procedure, the permeablization and intracellular staining was done prior to surface staining and no decrease in the percent of CD4 positive cells was detected (Figure 1b). The optimum concentration for Foxp3 was 20 µl of 1:200 dilution as obtained from the titration experiment. The mean percent of CD4+FoxP3+ cells was shown to be 0.81%.

Use of three-color Flowcytometry for the Detection of Circulating nTregs and the Analysis of the Frequency of nTregs in Chronic HCV Patients. Natural Tregs were defined as $CD4^+$ T cells expressing CD25 and FoxP3. Using the optimized method, the multiparametric analysis of cell surface expression of CD4, CD25 and intracellular expression of FoxP3 was performed on peripheral lymphocytes. A significantly higher proportion of Tregs in the total CD4+T cell population was seen in the peripheral blood of chronic HCV patients (0.83%) compared to the healthy controls (0.26%) (Figure 2).

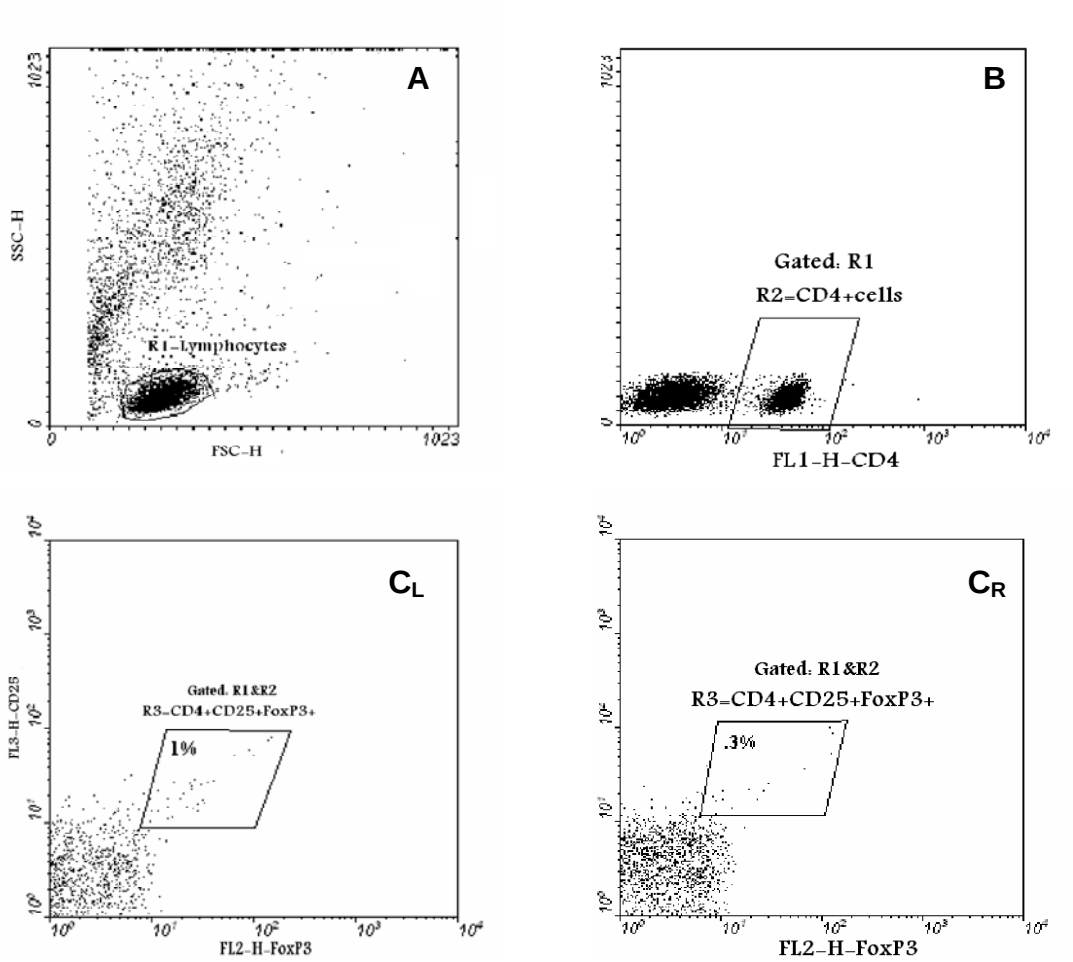

Figure 2. Representative staining characteristics and gating strategies are shown for CD4, CD25 and FoxP3 T-cell subsets in PBMC A) R1 is the population of lymphocytes which has been selected for detection of $CD4^+$ cells to exclude the non-lymphocytes (gating on lymphoid cells based on forward and side scatter characteristics). B) CD4 T lymphocytes (R2) which drieved from R1. C) Increased $CD4^+CD25^+FoxP3^+$ nTregs frequency in HCV-infected subjects (left) comapared to control ones (right).

DISCUSSION

This study represents an in-house altered method using non-commercial buffers which permits the detection of Tregs, in a complex population of PBMCs using a small volume of blood. In general, it is not routine to use a fixative solution for the detection of surface markers, but we used it to detect both surface and intracellular markers simultaneously. Fortunately, our experiments showed that the fixation process did not destroy epitopes, because with an increase in the dilution of mAbs, a higher percentages of CD4/CD25 positive cells. Moreover, PFA may cause some increase in background fluorescence, but we used isotype controls for each markers to eliminate the background noise. Regarding this fact that the relative concentrations of antibody and antigen control the extent of antibody-antigen complex formation, different concentrations of each antibody were tested to find the optimal frequency for each marker. Fc blocking is important to prevent nonspecific binding common to many cell types, including B lymphocytes, natural killer cells, granulocytes, monocytes, macrophages and platelets (26), but as our experiments showed, it seems that for T lymphocytes this step can be omitted. For combined detection of surface antigens and the intracellular molecules, two different procedures were tested. In routine protocols for the simultaneous detection of surface and intracellular markers of Tregs, surface molecules are usually the first markers to be stained before performing triton permeabilizing of the cells for intracellular staining (1,27). However, this type of permeabilization procedure may result in the loss of surface molecules (28). To test the influence of triton permeabilizing procedure on the detection of CD4+ cells in PBMCs, two different scenarios were considered. In the first setting, PBMCs were fixed and stained by an appropriate dilution of anti-CD4 antibody. After washing, cells were permeabilized and incubated with monoclonal FoxP3-PE antibody. The results showed that triton treatment caused a sharp decrease in CD4 frequency by modification of antibody binding capacity (ABC). Consequently, the percentage of CD4 T cells detected following permeabilization was about 20% in different tests. Such a dramatic effect of triton treatment was confirmed when the procedure was repeated with both surface molecules (data not shown). This result was similar to a previous study which reported that triton treatment modifies the CD4 MFI significantly (26). In the second setting, cells were permeabilized and stained with monoclonal FoxP3-PE antibody and then incubated with anti-CD4 antibody. This study revealed that the staining of the surface molecules following cell permeabilization left the CD4 and CD25 frequency unchanged. Using the optimized method, the frequency of $CD4^+$ cells (50%) (29), $CD25^+$ cells (1.46%) (28,29), $CD4^+Foxp3^+$ cells (0.81%) and nTregs (0.26%) (1) in healthy individuals were in accordance with the previously reported data. In the present study consistent with most previous reports (2,5,19,22), an elevated frequency of Tregs in peripheral blood from patients with chronic HCV infection compared to healthy controls was shown. Several parameters may contribute to the discrepancy between the present data and those with no increase in the level of nTregs. These include differences in patient profiles, stage and the identification method of circulating Tregs. While the complete mechanisms are not clear, $CD4^+/CD25^+$T cells seem to play a major role in the control of Th1 mediated immune responses, which are the main mediators for HCV disease resolution (30). Alternatively, Tregs may also protect the host from liver destruction by suppressing immunoreactive T cells (25). However, we observed that the frequency of nTregs in chronic hepatitis was not related to the grade of

inflammation or the serum level of ALT (data not shown).
In conclusion, the in-house procedure of nTreg staining is only possible if the cells were permeabilized before surface staining. This method permits the concomitant detection of surface and intracellular antigens within target cells. It also permits the identification of nTregs and also the difference between the frequency of nTregs in patients and normal individuals. This assay is rapid, simple, reliable and cheap for further investigation on the identification of the possible roles of nTregs in different stages of the disease and for the effect of viral antigens in the increase of Tregs. Using this technique, we observed an elevation of Treg cells in chronic HCV infection, and this may play an important role in viral persistence by modulating virus-specific immune responses. In light of these findings, Treg cells may represent a potential target for the treatment of chronic HCV.

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