Original Article

Iranian Journal of Immunology

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Rosuvastatin Intervention Decreased the Frequencies of the TIM-3+ Population of NK Cells and NKT Cells among Chronic Hepatitis B Patients

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ABSTRACT

Background: Natural killer (NK) cells are dichotomously involved in chronic hepatitis B (CHB) infection as principal members of innate immunity. An effective treatment should enhance the antiviral potentials of NK cells and not their immunomodulatory roles. TIM-3 (T-cell immunoglobulin and mucin-containing domain) is a molecule with an essential role in controlling immune tolerance. TIM-3 demonstrated the highest expression among NK cells of patients with chronic liver disorders. Statins have been reported to attenuate the levels of TIM-3 on NK cells.

Objectives: To investigate the frequencies of NK cells, NKT cells, and TIM-3+ population in patients with CHB upon rosuvastatin (RSV) intervention.

Methods: Thirty confirmed patients with CHB were randomly assigned into two groups of 15 (receiving 20 mg of RSV or placebo per day) for 12 weeks. We evaluated the percentages of TIM-3+ cells by staining the peripheral blood mononuclear cells (PBMCs) with CD3, CD16, and CD56 markers using flow cytometry.

Results: Our findings indicated that RSV administration could increase CD3- CD56+ NK cells ($P>0.05$) and CD3+ CD16+ CD56+ NKT cells ($P<0.05$). RSV intervention could reduce the percentages of TIM-3+ cells among NK cells ($P<0.01$) and NKT cells ($P> 0.05$) of patients with CHB compared with the placebo group.

Conclusions: The increased population of NK and NKT cells and the effective reduction of TIM-3+ cells among patients with CHB delineated that rosuvastatin could be proposed as an appropriate modulator of innate immune response (regarding NK and NKT cells) in favor of enhancing their antiviral activities.

Keywords: Rosuvastatin, HBV, TIM-3, NK cells, NKT cells

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Cite this article as:
Norouzi AR, Taziki S, Najafipasandy A, Mohammadi S, Roshandel GR. *Rosuvastatin* Intervention Decreased the Frequencies of the TIM-3+ Population of NK Cells and NKT Cells among Chronic Hepatitis B Patients. *Iran J Immunol.* 2022; 19(3):255-262, doi: 10.22034/iji.2022.93726.2250.

Received: 23-01-2021
Revised: 26-02-2022
Accepted: 15-03-2022

INTRODUCTION

Hepatitis B virus (HBV) infection results in a propagated chronic disease involving heterogeneous manifestations, including liver damage, hepatocellular carcinoma (HCC), and cirrhosis (1). Although the vaccination of newborns is still considered the dominant strategy against HBV to eliminate unfavorable, long-lasting complications of chronic disease, several factors have decelerated vaccination programs, especially in the developing countries (2). The pathogenesis of chronic hepatitis B (CHB) is crucially attached to the maturity of the immune system and the interaction between the virus and distinct antiviral immune cells, including Kupffer cells (KCs), dendritic cells (DCs), and natural killer (NK) cells (3). The effector cells' ability to produce inflammatory cytokines, such as IFN-γ, IL-6, IFN-α, and IL-1, is impaired (4, 5), thus intending to prompt a tolerogenic immune response to produce immunomodulatory molecules (6). Since the CD4/CD8 T cell response is defective (7) and innate immunity is also weakened (8), viral replication remains continuously high in CHB patients (9). Consistently, modulators of the immune response, especially those triggering innate immune cells, have been suggested as potential therapeutic approaches against CHB (10). Current antiviral therapeutics against CHB fall into two main categories of nucleotide analogs (such as entecavir, adefovir, tenofovir, and lamivudine) or interferons (such as IFN-a2b) (11). Although these drugs can efficaciously subdue HBV replication and decrease the morbidity among CHB patients (12), in favor of promoting T cells mediated immune response, the efficacy of such drugs remains controversial in respect to innate immune response (13).

Natural killer (NK) cells are among group I innate lymphocytes (ILCs) within the innate immune system, with significant roles against viral infections (14). In cooperation with other variables, NK cells are also firmly involved in maintaining hepatic tolerance, which is detrimental to an effective anti-viral immune response (15). The expression of multiple NK cell surface markers, responding to environmental stimuli such as cytokines, balances their functions (16). NK T (NKT) cells are a subgroup of T cells expressing markers related to NK cells that mediate immune response against viral infections, including CHB (17). T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) is a protein expressed on the surface of NK cells and NKT cells (18, 19). According to their ligand (galectin-9) expression by the surrounding Kupffer cells, the upregulation of TIM-3 could damage their function among CHB patients (19). The overexpression of TIM-3 in monocytes and NKT cells of CHB patients was reported to be associated with elevated liver function enzymes and decreased pro-inflammatory cytokines such as TNF-α (20).

Rosuvastatin (RSV) is a statin (HMG-CoA reductase inhibitor), basically involved in balancing the lipid profile, which possesses potential immunoregulatory effects (21). It has been suggested that statins, including RSV, can reduce TIM-3 expression in NK and NKT cells of atherosclerotic patients (22).

Regarding the significance of NK and NKT cells in the pathogenesis of CHB, the dominant role of TIM-3 in exerting the immunomodulatory effects of innate immune cells against viral infections, and the suggested positive effects of RSV in favor of enhancing the immune response, we investigated the impact of RSV intervention on expanding/shrinking the NK and NKT populations, and also assessing the frequency of TIM-3+ NK/NKT cells in CHB patients.

MATERIALS AND METHODS

Patients and Study Design

Thirty CHB patients (matched according to gender, age, and other treatment strategies) were prospectively recruited from the Gastroenterology and Liver Clinic, Sayyad-

e-Shirazi Hospital, Golestan University of Medical Sciences (GoUMS), Gorgan, Iran. The presence of CHB was initially clinically and experimentally confirmed in all patients. Briefly, the hepatitis B surface antigen (HBsAg) was positive in all the participants on at least two asynchronous stages in the last six months, with HBV DNA over 105 copies/mL. The elevated serum levels of alanine aminotransferase (ALT) were steadily observed in all the participants for the previous six months. Patients with HAV, HCV, HDV, or HIV co-infections were omitted. Moreover, all participants with non-viral chronic liver damage, acute renal failures, autoimmune disorders, malignancies, drug or alcohol dependence, pregnancy, and history of hypersensitivity to statins were excluded. All the patients signed their written informed consent after receiving detailed information about the benefits and possible side-effects of participation in this study. The Ethics Committee at GoUMS approved the study protocols and confirmed the research guidelines by the declaration of Helsinki (Code of Ethics: IR.GOUMS.REC.1397.342).

Registration, Randomization, Study Protocols, and Clinical Interventions

The study was a prospective, single-centered, randomized, double-blinded, placebo-controlled clinical trial to evaluate the outcome of rosuvastatin treatment on the levels of TIM-3 among NK cells and NKT cells of CHB patients. The trial was registered before the recruitment of the patients on the Iranian Registry of Clinical Trials (Code: IRCT20190602043789N1). The patients were randomly assigned into two groups of 15 (1:1 ratio) by an individual not responsible for this research, either receiving rosuvastatin or a placebo. The rosuvastatin pills and/or placebo counterparts, identical in physical appearances, were placed in enclosed packages by a non-affiliated pharmacist. The patients', health caretakers, and clinical and experimental investigators were blinded to the assignment of participants. Rosuvastatin 20 mg tablets (Abidi, Iran) or the placebo were orally prescribed for all participants once and daily for up to 12 weeks. Clinical and laboratory parameters were measured before and after the accomplishment of the interventions. Any possible adverse effects were recorded, consulted with the specialist, and the interventions were discontinued in case of contradiction with the study protocols. Finally, five ml. of peripheral blood was taken from all the participants and aliquoted in sterile tubes (with and without anticoagulants). Samples were then delivered to the Stem Cells Research Center Laboratory at GoUMS.

Immunophenotyping of NK Cells and NKT Cells by Flow Cytometry

PBMC isolation was performed using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation. After confirming the viability of PBMCs, 2-5×10^5 cells were resuspended in freshly prepared warm PBS for further staining. The staining of PBMCs was conducted using FITC-CD3 (Cat # 300306; Biolegend, San Diego, USA), PE-CD16 (Cat # 302008; Biolegend), PE/Cy7-CD56 (NCAM) (Cat # 362510; Biolegend), and APC-CD366 (TIM-3) antihuman antibodies. We utilized BD Accuri C6 flow cytometer (BD PharMingen, San Diego, USA) and BD Accuri C6 plus software to measure the immunophenotypes of all samples.

Statistical Analyses

We analyzed the data using SPSS 23 and GraphPad Prism 8 statistical software. To compare the differences between the two groups, the Independent Samples t-test was employed. P-values lower than 0.05 were appraised as statistically significant.

RESULTS

Rosuvastatin Administration Increased CD3- CD56+ NK Cells and CD3+ CD16+ CD56+ NKT Cells among CHB Patients

We gated the lymphocyte populations among PBMCs (Figures 1A and 1E) and quantified the percentages of CD3- CD56+ (NK) cells among both groups of CHB patients (placebo and rosuvastatin). Although not statistically significant, Figure 1I demonstrated that RSV treatment elevated the percentages of CD3- CD56+ NK cells among CHB patients (flowcytometric scatter plots: Figures 1C vs. 1G). Likewise, we assessed the frequencies of CD16+ CD56+ cells on CD3+ lymphocytes in both the treatment groups of CHB patients (Figures 1D vs. 1H). As shown in Figure 1J, RSV intervention significantly increased the percentages of CD3+ CD16+ CD56+ (NKT) cells in comparison with the placebo group (P<0.05).

Rosuvastatin Reduced the Percentages of TIM-3+ Cells among NK Cells of CHB Patients

We compared the percentages of TIM-

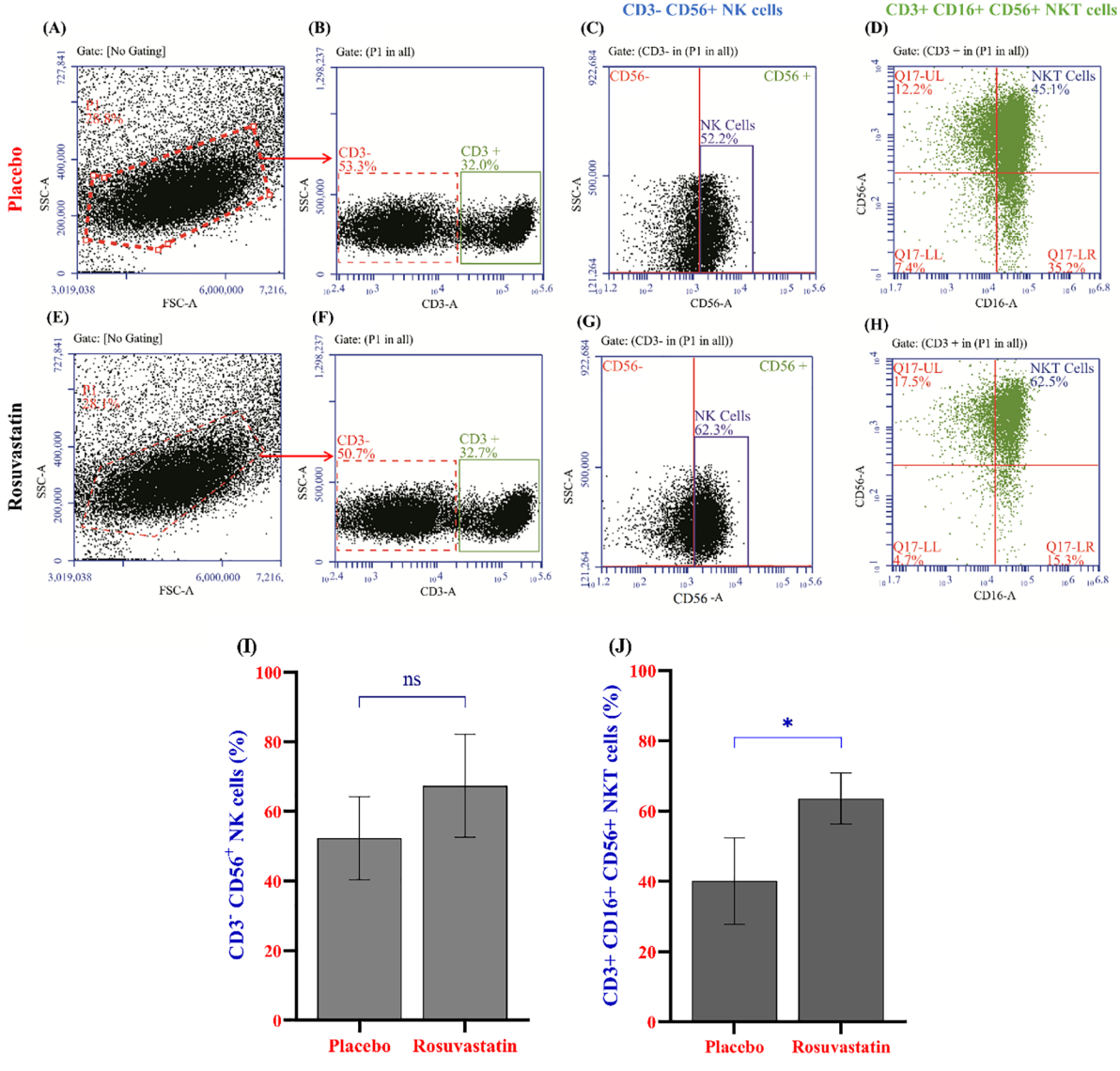

Figure 1. Immunophenotyping of CD3- CD56+ NK cells and CD3+ CD16+ CD56+ NKT cells in chronic hepatitis B (CHB) patients treated with rosuvastatin or placebo. FITC-conjugated anti-human CD3 Antibody, PE-conjugated anti-human CD16 Antibody, and PE/Cy7-conjugated anti-human CD56 Antibody were used to stain gated lymphocytes (A-G). Rosuvastatin treatment increased the percentages of CD3- CD56+ NK cells among CHB patients (I). Rosuvastatin intervention could significantly increase the percentages of CD3+ CD16+ CD56+ (NKT) cells in comparison with the placebo group (J). Independent Samples t-Test was used to compare the means between the two groups. Each bar represents Means±Standard deviation. Ns: not significant; *P value<0.05.

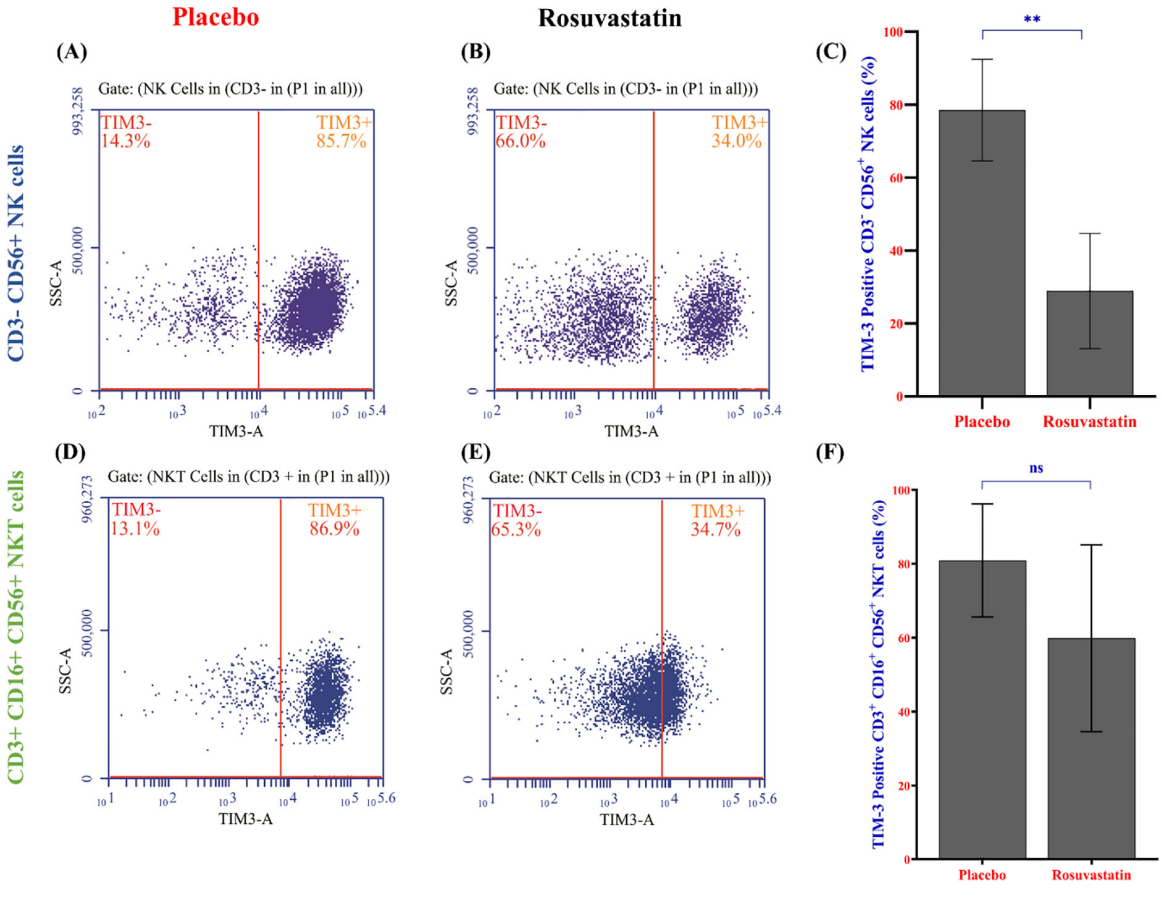

Figure 2. Evaluating the percentages of TIM-3+ cells among NK cells and NKT cells of CHB patients treated with rosuvastatin or placebo. We found that RSV intervention could markedly reduce the percentages of TIM-3+ cells among NK (A, B, C) and NKT (D, E, F) cells in CHB patients. Independent Samples t-Test was used to compare the means between the two groups. Each bar represents Means ± Standard deviation. Ns: not significant; **P value<0.01.

3+ cells among CD3- CD56+ NK cells of RSV and placebo-treated CHB patients (Figures 2A and B). As shown in Figure 2C, rosuvastatin intervention significantly declined the percentages of TIM-3+ cells among NK cells of CHB patients (P<0.01).

Administration of RSV Decreased TIM-3+ Cells among NKT Cells

We also evaluated the percentages of TIM-3+ cells among CD3+ CD16+ CD56+ NKT cells of RSV and placebo-treated CHB patients (Figures 2D and E). As shown in Figure 2F, RSV administration could markedly decline the percentages of TIM-3+ cells among NKT cells of CHB patients. However, the decrease was not statistically significant.

DISCUSSION

CHB viral infection is a major public health issue worldwide, responsible for liver-associated severe complications, including cirrhosis (23). Although most patients are successfully treated with current medications, untreated CHB patients remain susceptible to developing irreversible liver failure (24). The maturity of the immune system and functionality of related operating cells determine the effectiveness of the immune response in favor of being either antiviral or tolerogenic (3, 6, 25). Despite the documented proof of the inadequate adaptive immune response, the consequence of a weakened innate immunity is considered irrecoverable and also suitable for the constantly high viral

replication in CHB patients (26).

Natural killer (NK) and NKT cells are known as "double-edged swords" of the innate immunity against viral liver infections, which may give rise to the antiviral immune response or support developing a tolerogenic milieu in cooperation with other effector cells and molecules (14, 15, 27). TIM-3 is a surface marker majorly expressed on NK cells and NKT cells (18, 19). Galectin-9, usually defined by Kupffer cells, is introduced as the specific ligand for TIM-3 (19). TIM-3/galectin-9 signaling pathway has been suggested to mediate the suppression of NK cell function and increase the risk of developing HCC in CHB patients (28, 29). Recent findings indicated that TIGIT+ TIM-3+ NK cells were involved in the functional exhaustion of NK cells and disease progression of HBV-related HCC (30). It has also been reported that TIM-3 was upregulated in the monocytes and NKT cells of CHB patients in association with the impaired LFTs and deregulated inflammatory immune response (20).

Statins (HMG-CoA reductase inhibitors), including rosuvastatin (RSV), are primarily involved in stabilizing normal lipid profiles (21). However, several immunomodulatory effects of such compounds have been reported (31), while RSV may reduce the expression of TIM-3 molecules on NK and NKT cells of atherosclerotic patients (22). In the present study, we conducted a prospective, single-centered, randomized, double-blinded, placebo-controlled clinical trial to evaluate the effects of RSV intervention on expanding/shrinking the NK and NKT populations and assess the frequency of TIM-3+ NK/NKT cells in CHB patients. Our findings revealed that RSV administration could increase CD3-CD56+ NK cells and CD3+ CD16+ CD56+ NKT cells among CHB patients. Previous studies have revealed that IL-2 co-stimulation may enable the statin-mediated activation of human NK cells through a mechanism involving CD56+ DCs (32). However, to the best of our knowledge, this is the first study reporting the successful effects of rosuvastatin on expanding the NK cells and NKT cell populations in CHB patients. We also found that RSV intervention could markedly reduce the percentages of TIM-3+ cells among NK and NKT cells in CHB patients. However, the decrease of TIM-3+ NKT cells was not statistically significant. Regarding the inhibitory effects of statins on the cytotoxic functions of NK cells, statins have been suggested to reduce the engagement of NK-cell receptors by target cell ligands by altering their polarization and adherence (33). Although the downregulation of TIM-3 in NK and NKT cells of atherosclerotic patients has been reported (22), this is the first study demonstrating this reduction among CHB patients, which could be attributed to the indirect antiviral effects of RSV. However, the exact mechanism is unclear and needs further study. Following our findings, Yakin et al. (2017) revealed that statins can reduce the risk of cirrhosis in hepatitis B or C patients (34). Although downregulation of TIM-3 is in favor of enhancing the antiviral immune response and better prognosis of CHB patients, a cohort study is needed. Following our findings, the immune response is limited in the tumor microenvironment and persistent inflammation, in which TIM-3 expression on NK cells and NKT cells increased (35-37). The current study was associated with several limitations, including a small sample size and a lack of information about the clinical status of the patients, which might affect the results. Moreover, measuring the alterations in the populations of NK and NKT cells at the time of recruitment (day 0), and comparing the results with the final day (day 84), could put on more compelling data.

In conclusion, the administration of RSV effectively expands the populations of CD3-CD56+ NK cells and CD3+ CD16+ CD56+ NKT cells and decreases TIM3+ NK and NKT cells among CHB patients. Although the increase of CD3- CD56+ NK cells and the decrease of TIM-3+ NKT cells were not statistically significant, our findings would help develop convenient antiviral treatment

strategies to control chronic hepatitis B infection.

RESEARCH INVOLVING HUMAN PARTICIPANTS

The present study, which involved clinical interventions in human participants (a double-blind placebo-controlled randomized clinical trial), was approved by the Ethical Committees of GoUMS (Code of Ethics: IR.GOUMS.REC.1397.342). An informed consent following the declaration of Helsinki was signed by all the participators. The trial was registered before the recruitment of patients on the Iranian Registry of Clinical Trials (Code: IRCT20190602043789N1).

ACKNOWLEDGMENTS

All data in this article was obtained from a residency thesis in the field of Internal Medicine written by Dr. Ali Najafi at Gorgan School of Medicine, Golestan University of Medical Sciences (GoUMS), Gorgan, Iran. This study was supported by Golestan University of Medical Sciences, Gorgan, Iran (Grant Number:110176).

Conflict of Interest: None declared.

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